COMPLEMENT ACTIVATION DOES NOT PLAY A DIRECT ROLE IN MODEL CHEMOTHERAPUTIC – INDUCED MUCOSITIS

by

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Dalhousie University is located in Mi'kma'ki, the ancestral and unceded territory of the Mi'kmaq. We are all Treaty people.

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ABSTRACT

Chemotherapeutic agents are highly efficient in the treatment of various cancers; however, many of the drugs also lead to unwanted toxicities. “Mucositis” describes the injury caused by these cytotoxic agents to the healthy mucosa of the digestive tract. Depending on the dose regimen and drug, mucositis afflicts a significant fraction of patients, from 40-100%. Currently, the underlying molecular mechanism(s) responsible for mucositis are not fully understood and there are no efficient treatments.

Research into the mechanism underlying mucositis has identified superoxide radicals early followed by changes in mediators of inflammation and subsequent tissue injury. It was previously reported that complement becomes activated in the jejunum of mice injected with 5-fluorouracil to elicit mucositis and that properdin deficient mice, which should have compromised alternative pathway activation, were protected though in a complement-activation-independent mechanism. While that discovery implicated properdin in the inflammation, the experiment did not rule-out other pathways of complement activation possibly contributing to the inflammation. Therefore, the objective of this study was to determine whether activation of complement by other routes contributes to mucositis, and the hypothesis; that preventing complement activation would protect mice from mucositis. Mice were injected daily with 5-fluorouracil or methotrexate for 5 days then euthanized one day later. Each mouse’s jejunum, colon, tongue and cheek mucosae were harvested for histopathological analysis. To determine whether the lectin pathway was involved, mice lacking mannose binding lectins 1 and 2 were used. Deficient mice responded similar to wildtype mice to 5-fluorouracil and methotrexate, showing weight loss at the same rate and similar pathological features in both the jejunum and colon. Neither strain of mouse manifested with oral mucositis.

Having ruled-out the mannose-binding lectin pathway, it was decided to use mice deficient in the molecule central to complement, C3. C3 deficient mice were bred with wild type mice, then the heterozygous offspring bred to a second generation which were used in experiments. The stool bacteria showed a pattern consistent with the F2 generation mice becoming more similar though different from their parent’s. C3 deficient and C3 wildtype drug-treated mice lost weight to a similar extent, while C3 heterozygotes lost weight beginning on the third day of the experiment. All the mice had similar histopathological features in their jejunums and colons after the treatments. No mice developed oral mucositis. One experiment extending the period of time the mice were treated was conducted but none of the mice developed oral mucositis. I conclude that complement activation does not contribute mechanistically to mucositis.

LIST OF ABBREVIATIONS AND SYMBOLS USED

5-FU	5-fluorouracil
AP	Alternative pathway
C3aR	C3a receptor
CIM	Chemotherapy-induced mucositis
COX-2	Cyclooxygenase-2
CP	Classical pathway
CRP	C-reactive protein
DAMP	Danger associated molecular pattern
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPD	Dihydropyrimidine dehydrogenase
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
fB	Factor B
fD	Factor D
FdUMP	Fluorodeoxyuridine monophosphate
FdUTP	Fluorodeoxyuridine triphosphate
FUTP	Fluorouridine triphosphate

G-CSF	Granulocyte-colony-stimulating factor
GIM	Gastrointestinal mucositis
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factors
H&E	Hematoxylin and eosin
HZ	Heterozygous
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IM	Intestinal mucositis
IP	Intraperitoneal
IR	Ionizing Radiation
ISC	Intestinal stem cells
IV	Intravenous
KO	Knockout
LP	Lectin pathway
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAC	Membrane Attack complex
MASPs	Mannose binding lectin-associated serine proteases
MBL	Mannose Binding Lectin
MTX	Methotrexate
MMPs	Matrix metalloproteinases
NAC	N-acetyl cysteine

NF-κB	Nuclear factor kappa-B
OM	Oral mucositis
PCR	Polymerase chain reaction
rhIL-1Ra	Recombinant human interleukin-1-antagonist
ROS	Reactive oxygen species
PBS	Phosphate-buffered saline
RNA	Ribonucleic Acid
sd	standard deviation
SPF	Specific Pathogen Free
THF	Tetrahydrofolate
TNF	Tumor necrosis factor
TS	Thymidylate synthase
WT	Wild type

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CHAPTER 1: INTRODUCTION

1.1. Chemotherapy-induced mucositis

1.1.1. Clinical considerations and symptoms

Chemotherapy-induced mucositis (CIM) in the digestive tract is the most common and clinically significant side effect of treating cancer patients (Cinausero et al., 2017, Sonis, 2004). General speaking, mucositis refers to the inflammatory and ulcerative lesions caused by anti-cancer therapies to the mucosa of the mouth, esophagus, stomach, and small and large intestines (Chang et al., 2012; de Koning et al., 2006; Jain et al., 2017; Sonis et al., 2004). Mucositis has been further subclassified based on anatomical location: oral mucositis (OM) for lesions in the oral cavity including the buccal mucosa and tongue, and gastrointestinal mucositis (GIM) for lesions in the stomach and intestines. Damage specific to the intestines is referred to as intestinal mucositis (IM) (Cinauseroet al., 2017; Keefe, 2007). Severe OM and IM are responsible for significant morbidity in cancer patients (Peterson & Cariello, 2004; Lima-Júnior et al., 2014; Sonis et al., 2001; Spencer, 2016).

Studies estimate that mucositis affects approximately 40% of cancer patients receiving standard doses of chemotherapy and 100% of patients receiving high doses (Keefe, 2004; Lima-Júnior et al., 2014; Sonis, 1998; Sonis, 2004; Yeoh et al., 2005). Sixty to 85% of patients undergoing hematopoietic stem cell transplantation will experience mucositis (Villa & Sonis, 2015). Patients may suffer from one or both conditions with a higher rate (>90%) of OM in children (Bresalier & Davila, 2008; Eltinget al., 2003; Huang et al., 2019).

A number of anticancer medications, including 5-fluorouracil (5-FU), doxorubicin, bleomycin, cisplatin, cyclophosphamide, carboplatin, etoposide, methotrexate (MTX),

busulfan, selected tyrosine kinase inhibitors, and epidermal growth factor receptor (EGFR) inhibitors all lead to mucositis in some patients (Oronskyet al., 2018). Yet, not all anticancer agents are equipotent at causing mucositis. MTX, 5-FU, irinotecan (Abdel-Rahman et al., 2016; Mayo et al., 2017; Schwab et al., 2008; Stein et al., 2010; Zhang et al., 2018a), anthracyclines and taxanes (Kwon, 2016), cyclophosphamide and cisplatin (Villa & Sonis, 2015), all are more consistently linked with mucosal toxicities and higher risk of mucositis than hydroxyurea, bleomycin or etoposide. Finally, mucositis is not limited to drug treatments. Direct mucosal toxicity can occur from ionizing radiation (IR) applied to the head and neck (Oronskyet al., 2018). Clearly, mucositis is a concerning complication of cancer therapy and deserving of more attention.

The symptoms of OM range in severity from grade 1 (mild with redness and soreness) to grade 4 (preventing oral intake) (Oronskyet al., 2018). Grades 3 and 4 OM are associated with severe limitations in the patients' ability to swallow, drink, taste, eat, and speak, leading to malnutrition, dehydration, and often requiring narcotic analgesics for controlling pain, parenteral nutrition, or supplementary nutrition (Al-Dasooqiet al., 2013; Fiorentino et al., 1991; Sonis, 2004; Trotti et al., 2003). Symptoms include a large spectrum of digestive manifestations including diarrhea, bloating, vomiting, nausea and abdominal pain (Al-Dasooqi et al., 2013; Benson et al., 2004; Keefe et al., 1997; Keefe et al., 2007; Sonis, 2004). Both OM and IM can manifest as systemic symptoms such as anorexia, neutropenia, infections, sepsis due to bacterial translocation, and fatigue due to malnutrition and malabsorption (Aprile et al., 2008; Benson et al., 2004; Keefe et al., 1997; Keefe & Gibson, 2007; Sonis, 2004). Therefore, mucositis can further adversely impact the cancer patients' quality of life and is associated with multiple negative symptomatic, health and economic outcomes (Cinauseroet al., 2017; Sonis, 2004). Considering the impact of

mucositis on the patient's health, it can be a dose-limiting toxicity for cancer treatment due to drug dose reductions or treatment delays which directly reduce the efficacy of their anti-cancer therapy (Bertolini et al., 2017; Blijlevens et al., 2000; Lima-Júnior et al., 2014; Oronsky et al., 2018 ; Sonis, 1998; Sonis et al., 1999; Sonis et al., 2001; Sonis et al., 2004).

Cancer patients differ in their individual risk for mucositis (Cinauseroet al., 2017; Sonis, 2009). Some patients only experience minor side effects, while others experience significant severe consequences that require them to stop receiving cancer treatment (Chang et al., 2012). The variability of responses among patients can be attributed to the biologic complexity of mucositis (Sonis et al., 1999). Children have a higher rate of basal cell proliferation and a higher proportion of proliferating cells with the result that children are more prone to develop mucositis than adults (Sonis, 1998; Sonis et al., 1999). Patients who receive chemotherapy typically experience their initial symptoms shortly (3-5 days) after treatment, peaking between 7 and 14 days and recovering within the following week (Al-Ansari et al., 2015; Sonis, 2009; Villa & Sonis, 2015). Contrarily, radiotherapy-induced mucositis has a chronic course where patients are exposed to increasing doses during treatment and signs of mucositis typically appear during the second or third week of therapy, with ulceration lasting for 2-4 weeks following the last dose (Ribeiro et al., 2016; Sonis, 2009; Villa & Sonis, 2015).

1.1.2 Intestinal and oral cell anatomy

The small and large intestines have in common a simple columnar epithelial cell lining, but the 2 organs have some significant differences in anatomy, function, and cellular and microbial composition (Bowcutt et al., 2014). The mucosa is formed by the epithelium and lamina propria while the muscularis mucosa separates the mucosa from the submucosa, the muscularis propria and the serosa (Al-Dasooqi et. al, 2010) (Figure 1.1). For conducting

the principal function of digestion and nutrient absorption, the mucosa in the small intestine is folded into finger-like projections, perpendicular to the lumen direction. Villi are lacking in the mucosa in the large intestine. The columnar epithelial cells are joined by tight junctions on the apical lateral borders and by desmosomes on the lateral boarder, with the tight junctions providing most of the control over the monolayer permeability (Ceredaet al., 2018; Corrêa et al., 2017). Crypts in both intestines include intestinal stem cells (ISC) and in the small intestine, Paneth cells (Barker et al., 2012; Meran et al., 2017). All epithelial cells are replenished by daughter cells of the dividing ISC, migrating downward in the crypt to become Paneth cells, or upwards, first into a zone of "transit amplifying cells", while differentiating into the other cell types (Sonis, 2004). Final differentiated cell types fall into one of two categories, secretory (Paneth, goblet, enteroendocrine and Tuft cells) or absorptive cells (enterocytes) (Bresalier & Davila 2008; Clevers & Bevins, 2013; Delgado et al., 2016; Peterson & Artis, 2014; Potten et al., 1997; Ratanasirintrawoot & Israsena, 2016; Strzyz, 2019). A high cell turnover rate results in the replacement of the entire epithelium each week, with effete cells undergoing anoikis (detached-induced cell death) and sloughing at the villus tip or the crypt surface in the colon (Al-Dasooqi et al., 2010; Grabinger et al., 2014). The process of cell division and differentiation is highly controlled by intrinsic and extrinsic factors (Booth, & Potten, 2001; Clevers & Bevins, 2013; Sato et al., 2010; Van Es et al., 2010). Nestled between some epithelial cells are CD3+ lymphocytes, "intraepithelial lymphocytes", with incompletely defined roles.

Beneath the epithelium, the lamina propria is a loose layer of connective tissue (Hahn et al., 1990; Pender & MacDonald, 2004; Wheatley, 2003). Leukocytes are abundant in the lamina propria, including macrophages, dendritic cells, and various lymphocytes (Mestecky et al., 2005; Peterson & Artis, 2014). The lamina propria constituents differ

throughout the gastrointestinal tract (GIT) where the lamina propria of the small intestine has a high concentration of lymphocytes (Cutler, 1990). The muscularis mucosa is a layer of smooth muscle cells demarcating the boundary between the mucosa and submucosa. The submucosa is composed of loose connective tissue, stromal cells and extracellular matrix, and dense irregular connective tissue, through which the main blood and lymphatic vessels pass (Clark et al., 2008). Overall, the intestines are fashioned into a long tube with the mucosa on the inside followed by the submucosa then the circular and longitudinal smooth muscle layers, the muscularis propria. The muscularis propria in turn is wrapped by the serosa.

The healthy oral mucosal epithelium is made up of a renewing keratinizing stratified squamous epithelium, lamina propria, and skeletal muscles layer (Figure 1.2). New cells are continuously produced by replicating basal stem cells juxtaposed to the lamina propria, to replace sloughing surface epithelial cells. The capacity of the tissue to regenerate continually is necessary for the continuity and structure of the normal oral mucosa. Cells move from the stem cell source to the surface in roughly 2 weeks (Sonis, 2004). Epithelial cells of the oral mucosa turnover typically every 7 to 14 days, more slowly than in the intestines, but explaining the susceptibility of the mucosa to CIM (Barasch & Epstein, 2011; Oronsky et al., 2018).

The oral mucosa is divided into three types. The "lining mucosa" tissue, which is the most abundant, exposed, and is more susceptible to trauma, covers the surfaces of the cheeks, ventral tongue, soft palate, and lips. The "masticatory mucosa", is found on the hard palate and gingiva. On the dorsal surface of the tongue, are multiple papillae, taste buds, that have sensory nerve endings for taste. The masticatory mucosa rate of turnover is longer than the lining mucosa, likely as a result of differences in thickness (Sonis, 2004).

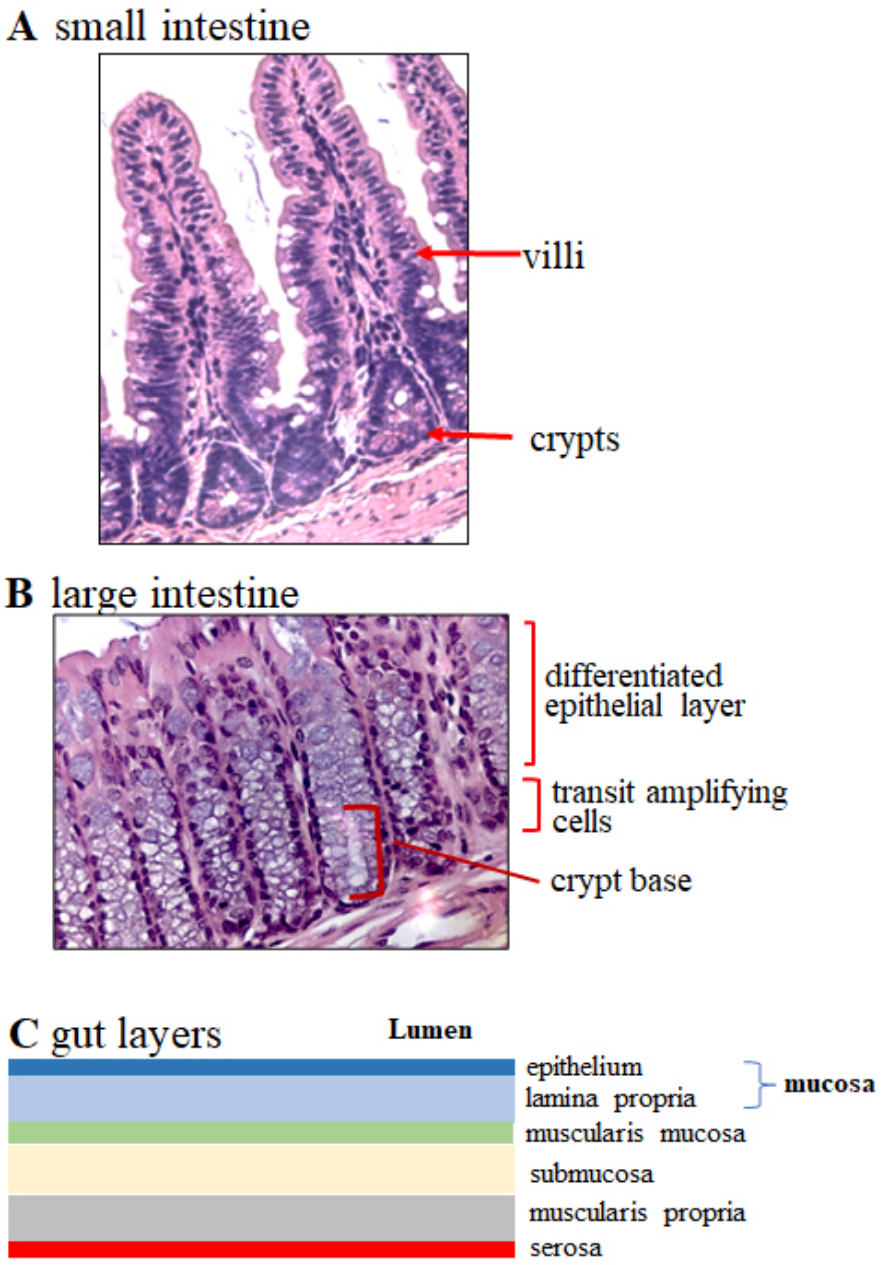


Figure 1.1 Histological architecture of the intestines. Stem cells that reside in the crypt divide rapidly to produce transit amplifying cells which migrate and differentiate into mature and functional cell types. **A** The mucosa of the small intestine is folded into villi, finger-like projections into the lumen, while **B** The large intestine lacks villi, leaving only crypts. **C** The fundamental layers of both intestines are the same. The muscularis propria includes circular and longitudinal smooth muscle layers. (The photomicrographs are originals).

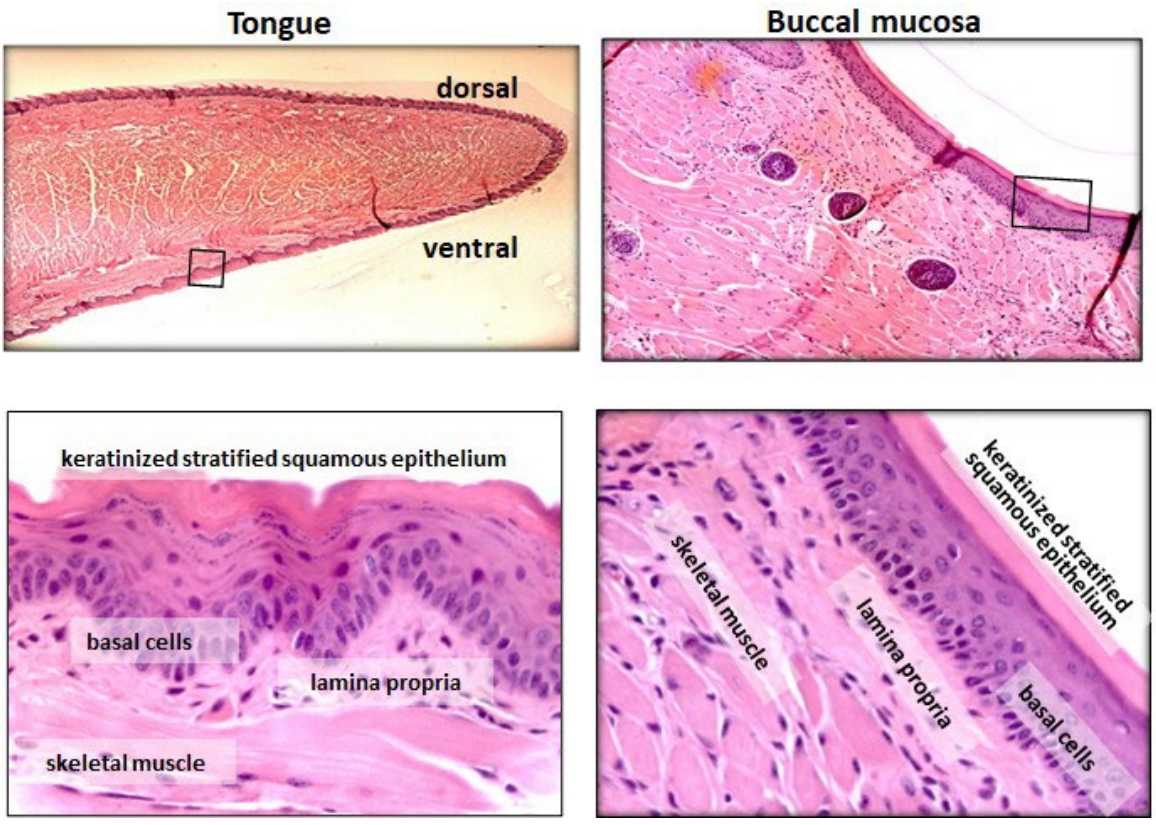


Figure 1.2 Histological architecture of the oral mucosa. Tongue (left panels) and buccal mucosa (right panels). Both tissues have in common keratinized stratified squamous epithelium, lamina propria, and skeletal muscles layers. Basal cells reside at the base of epithelium. The box in the top panels illustrate the section in the lower panels, under higher magnification. (The photomicrographs are originals).

1.1.3. Oral and intestinal mucositis histopathological characterization

OM and IM have common histopathological features (Cinausero et al., 2017). The impact on the small intestine and colon morphology is distinguished by shortening of villi, damaged crypts, enterocyte hyperplasia (Cinausero et al., 2017), decreased crypt length (Cinausero et al., 2017), increased apoptosis, epithelial atrophy (Gibson et al., 2005), degenerative enterocytes (Gibson et al., 2005; Logan et al., 2008), mucus hyposecretion (Stringer et al., 2009), damaged submucosal vasculature (Paris et al., 2001), leukocyte infiltration (Logan et al., 2008) and ulceration (Keefe, 2004; Sonis, 2004). OM is characterized by mucosal atrophy, disorganized epithelial structure, erythema, necrosis, and ulceration (Li et al., 2022). Regarding OM, in a study after treatment with 5-FU or bleomycin, mice showed increased cellularity of the subepithelial tissue, vascular dilatation, and leukocyte margination (Sonis, 1998). Endothelial cells and connective tissues are thought to be the initial oral tissue responding to chemotherapy or radiation, based on similar studies conducted in rodents (Sonis et al., 1999). When hamster oral mucosal biopsies were taken at various times after irradiation, electron microscopic analysis revealed damage to the submucosal connective tissue and blood vessels (Sonis, 2004; Sonis et al., 2000). Damage to both structures occurred over a week before any epithelial injury. In cancer patients, erythema is the first clinical manifestation of OM, affecting the movable mucosa including buccal or labial mucosa, ventral tongue, floor of the mouth or soft palate. In most cases, lesions develop into painful ulcerations that are frequently covered by a pseudo membrane and paired with odynophagia, dysphagia, malnutrition and weight loss (Chaveli-López & Bagán-Sebastián, 2016). In patients with severe neutropenia, disruption of the intact oral mucosa may be linked to microbial colonization that may remain localized or spread (Chaveli-López, 2014; Chaveli-López &

Bagán-Sebastián, 2016).

To classify the severity of mucositis, a symptom assessment using clinical mucositis grading scales is widely used (Al-Dasooqi et al., 2010). However, unlike OM, there is no single scale that is approved worldwide for staging IM (Sonis et al., 2004). The inability to acquire sequential biopsies before, during, and after therapy and the relative inaccessibility of the intestines make it near impossible to accurately assess the extent of mucositis (Sonis et al., 2004). This has important ramifications for clinical evaluation of interventions. Currently, breath tests and sugar permeability tests have been used to determine mucositis development, while histopathological data is considered the gold standard for mucositis diagnosis (Al-Dasooqi et al., 2010).

1.1.4. Management of mucositis

The management of mucositis remains a challenge (Cinausero et al., 2017; Sonis et al., 1999). Nevertheless, researchers use multiple approaches to try treat mucositis, some aiming at broad targets, some aimed at a specific target, and others are currently in development. Major categories of approaches or products are: *antioxidant agents* (glutamine, amifostine, oral zinc supplement, vitamin E, N-acetyl cysteine (NAC)), *anti-inflammatories and cytokine inhibitors* (benzydamine, pentoxifylline, salicylates, interleukin inhibitors), *probiotics and antimicrobial agents, pain management* (morphine mouthwashes, doxepin rinse, transdermal fentanyl, tapentadol, gabapentin, pregabalin), *cytoprotective agents* (sucralfate, prostaglandin analogs), *growth factors* (palifermin, colony stimulating factors (G-CSF, M-CSF), *anti-apoptotic agents* (caspase-3 inhibitors), *physical strategies* (oral cryotherapy, laser therapy), and *natural remedies* (vitamin A, ascorbic acid, manuka honey, aloe vera) (Cinausero et al., 2017). Presumably, treatment priorities can be identified by an understanding of the molecular and cellular processes

leading to, and perpetuating the injury.

Most research in humans has been conducted on OM, likely due to frequency, accessibility and the impact of symptoms (Al-Dasooqi et al., 2013; Sonis et al., 2004). Otherwise, researchers have turned to small animal models to study all aspects of the pathobiology of mucositis, and for use as pre-clinical testing platforms (Cinausero et al., 2017; Keefe, 2007). Hamsters have been the animal of choice for studying chemically induced OM. Hamsters have a sizable cheek pouch, and their oral mucosa reacts to treatment much like humans (Sonis, 2004). Yet, to achieve OM lesions, mechanical, chemical, or radiation injury in addition to the chemotherapy drug is required in most rodents (Bertolini et al., 2017, Huang et al., 2022). Even despite an additional injury to provoke OM, differing extents of keratinization of the oral epithelium between animals results in some animals requiring higher doses of drugs to elicit the same degree of mucositis seen in humans. Furthermore, as a matter of convenience, rodents are typically injected in routes other than intravenous (IV), the route of drug administration in patients. Still, the constant development of models and evaluations of how animal results compare with those in patients will assist in providing essential information to optimize the pathway for development of new therapies.

1.2. Mechanisms of cell injury by chemotherapeutic drugs

Cytotoxic chemotherapeutic drugs affect cells with high proliferation rates (Xiang et al., 2011). In general, chemotherapy drugs interrupt the cell cycle, leading to apoptosis. Consequently, while apoptosis is presumed to not be inflammatory, massive numbers of apoptotic cells trigger inflammation which further harms dividing and non-dividing epithelial cells (Lockhart & Sonis, 1981). Only after drug levels subside does the injury begin to reverse and tissues heal.

Not all drugs are equally effective at killing cells with drugs that affect synthesis of DNA (S phase) being the most potent (Focaccetti et al., 2015; Sonis, 1998). The DNA antimetabolites 5-FU and methotrexate (MTX) both impact the S phase of the cell cycle and consequently are more stomatotoxic than drugs that are non-specific (Sonis, 1998). Upon entering the cell, 5-FU converts into 1 of 3 active metabolites including fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate **(**FUTP**) (**Cascone, 2021; Wigle et al., 2019**) (**Figure 1.3). FdUMP inhibits thymidylate synthase, which in turns inhibits conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), a nucleotide necessary for replication of DNA. Thus, 5-FU administration causes a dUMP shortage that leads to dTMP reserve depletion. The consequence of these changes is impaired replication and repair of DNA and a lethal accumulation of damaged DNA culminating in apoptosis mediated through the intrinsic pathway (Johnston et al., 2003; Wigmore et al., 2010). FUTP or FdUMP metabolites can be incorporated into RNA and DNA strands, respectively, interrupting synthesis, which further contributes to cell death (Johnston et al., 2003; Miura et al., 2010; Peters et al., 2002). MTX inhibits dihydrofolate reductase (DHFR) which catalyzes dihydrofolate (DHF) conversion to tetrahydrofolate (THF), necessary for synthesis of nucleoside thymidine, required for DNA synthesis **(**Cascone, 2021) **(**Figure 1.4).

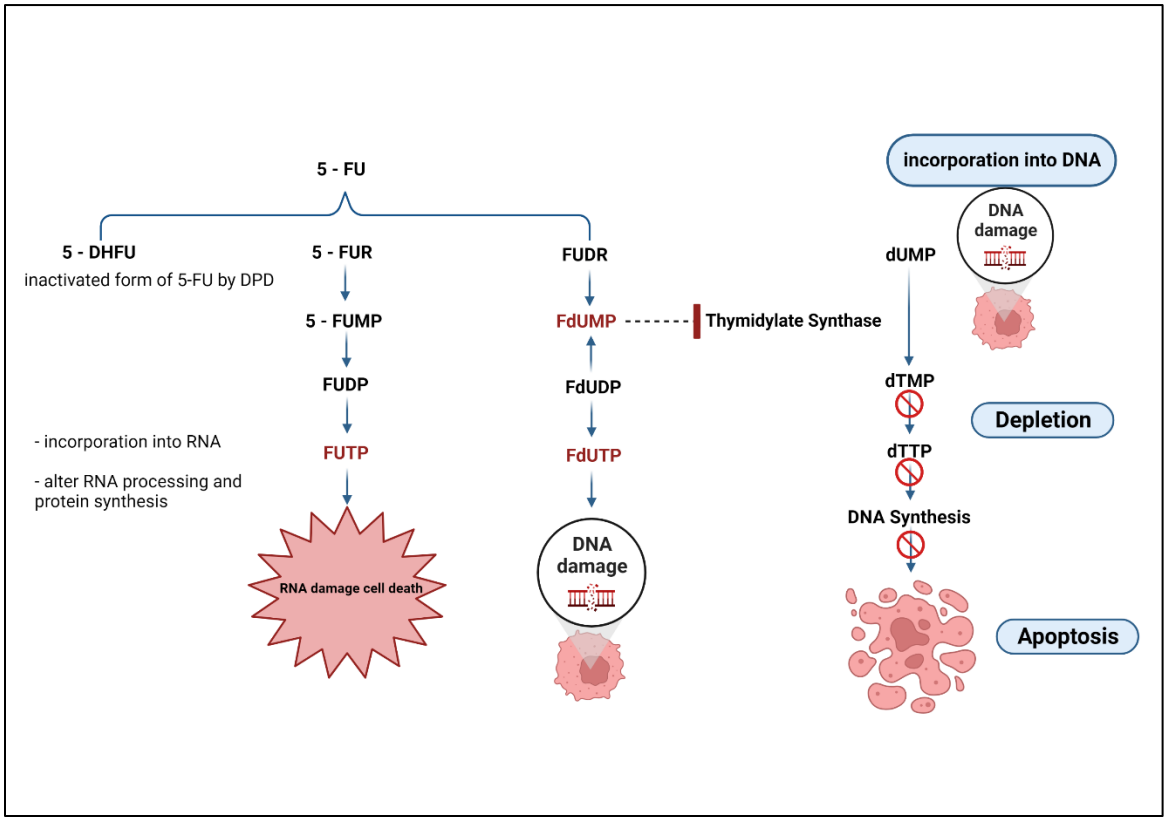


Figure 1.3 Mechanism of action of 5-fluorouracil. 5-FU converts to 3 active metabolites Fluoro-deoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), fluorouridine triphosphate **(**FUTP**),** through different enzymes. FUDR is converted by thymidylate phosphorylase to FdUMP which in turns blocks thymidylates synthase (TS) causing an imbalance of deoxyuridine monophosphate (dUMP) and deoxythymidine monophosphate (dTMP). FdUMP or FUTP metabolites incorporate into DNA and RNA respectively causing damage and leads to cell death (apoptosis). 5-DHFU is an inactivated form of 5-FU which is converted by dihydropyrimidine dehydrogenase (DPD).

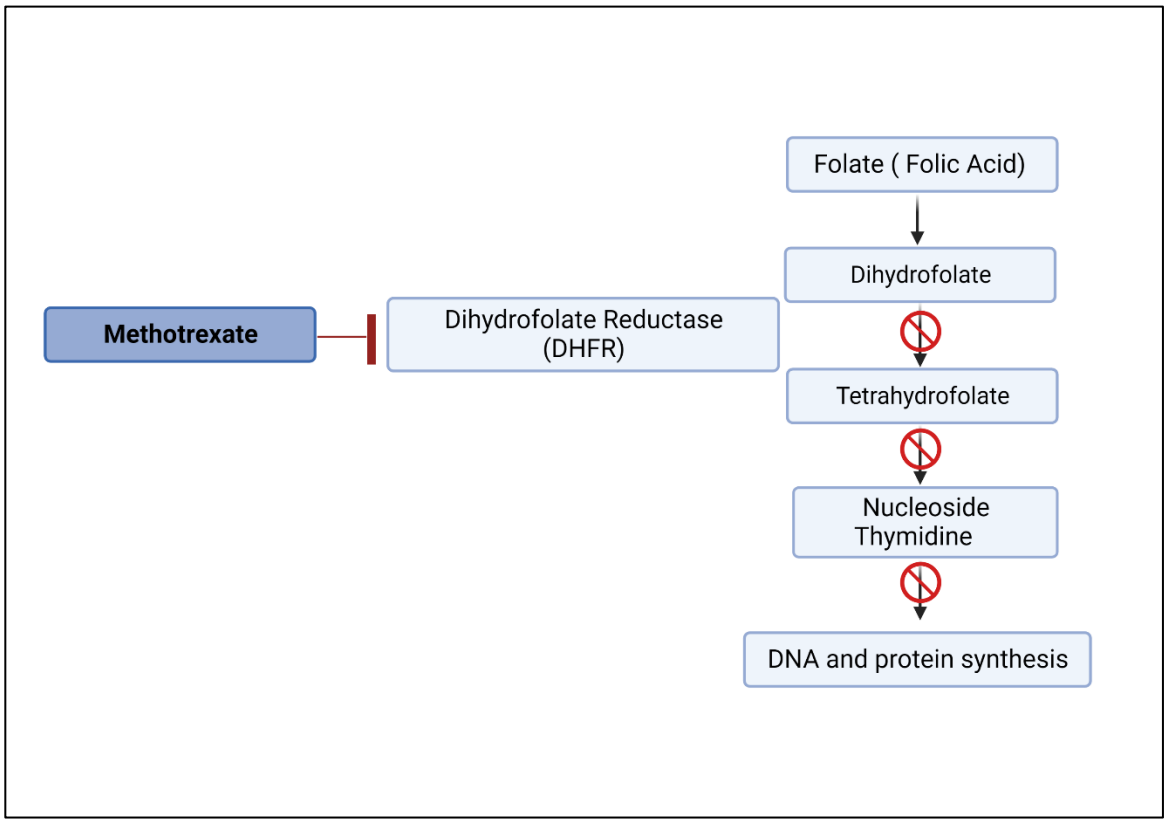


Figure 1.4 Mechanism of action of methotrexate. MTX blocks dihydrofolate reductase (DHFR), which converts dihydrofolate DHF to activated tetrahydrofolate THF, required for the nucleoside thymidine and DNA synthesis. Folate is essential for purine and pyrimidine base synthesis. Thus, MTX prohibits DNA, RNA, thymidylates and protein synthesis.

1.2.1. The 5-phase model for mucositis development

Notwithstanding that mucositis is a process that occurs continuously over time, Sonis has proposed 5 overlapping phases: ***initiation, message generation, signaling and amplification, ulceration,*** *and* ***healing*** (Figure 1.5) (Sonis, 1998; Sonis 2004; Villa & Sonis, 2015). ***Initiation*** includes the primary injury due to DNA and non-DNA damage, death of basal epithelial cells, reactive oxygen species generation (ROS), oxidative stress, and activation of the innate immune response. Chemotherapy triggers ROS production and lipid peroxidation that further triggers direct damage of DNA (Li et al., 2022). Lipid peroxidation results in release of cell membrane-bound molecules which increases immediate-response genes including c-JUN and c-JUN amino-terminal kinase. These pathways in turn activate and increase multiple transcription factors, like nuclear factor-kappa B (NF-κB) (Criswell et al., 2003; Davis, 2000). ***Message generation*** is characterized by NF-κB activation and upregulation of proinflammatory cytokines and chemokines (Huang et al., 2022). NF-κB activation can be a direct outcome of chemotherapy or activated secondarily by ROS (Logan et al., 2008). As a result of NF-kB activation many genes become expressed such as cyclooxygenase-2 (COX-2), inducible nitric oxide (NO)-synthase, superoxide dismutase and matrix metalloproteinases (MMPs), as well as proinflammatory cytokines like IL-1β, IL-6, and TNF (Abreu et al., 2010; Kwon, 2016; Logan et al., 2007; Logan et al., 2008; Sonis et al., 2004). Mediators like MMPs can disrupt mucosal integrity and cause mucosal destruction by thinning the epithelium. The ***signaling and amplification*** phase includes further cytokine production in a positive cytokine feedback loop that among other effects, further heightens activation of NF-κB. The result is an amplification and subsequent exacerbation and prolongation of tissue injury. Cytokines can increase the permeability of tight junctions in the epithelium (Allaire et al.,

2018; Al-Dasooqi et al., 2013; Marchiando et al., 2010). The product of this amplification is ***ulceration***, the clinical hallmark of mucositis. This phase is characterized by loss of mucosal integrity and epithelial erosion, resulting in painful ulcers. Ulcers act as an entry portal for bacteria and products of bacteria (cell wall antigens, α-glucans, lipoteichoic acid (LTA), and lipopolysaccharides (LPS), which penetrate through the submucosa into the circulation, causing bacteremia and sepsis (Peterson et al., 2010; Sonis et al., 2004; Sonis 2009; Villa & Sonis, 2015). Ulcers frequently develop secondary infections, particularly in patients experiencing neutropenia due to chemotherapy (Sonis, 2004). The presence of bacteria triggers activation of tissue macrophages and other inflammatory cells and infiltration to the base of the lesion, releasing additional damaging proinflammatory cytokines (Bain et al., 2013; Sonis et al., 2004; Thiesen et al., 2014; Villa & Sonis, 2015). As a result, small intestinal epithelial damage during CIM is characterized by villi atrophy, disturbed crypt cell homeostasis, increased apoptosis, disrupted intestinal permeability, reduced mucosal absorptive surface area, a reduction in mucin, dysbiosis, and hypo-proliferation (Carneiro-Filho et al., 2004; de Koning et al., 2007; Peterson & Cariello, 2004; Sonis et al., 2001). Mucositis is reversible in patients receiving chemotherapy upon chemotherapy cessation. ***Healing*** is characterized by an increase in the rate of epithelial cell proliferation, migration, differentiation, and restoration of the squamous epithelium in the oral cavity (Sonis, 2004; Sonis, 2009). Noteworthy, while the oral mucosa may recover during the healing phase, because of lingering angiogenesis, the patient is at higher risk for developing new episodes of mucositis (Al-Ansari et al., 2015; Blijlevens & Sonis, 2006). OM typically takes 4 days from the initial basal cell injury until there are clinically obvious mucosal alterations (erythema and thinning), and ulceration follows soon after. IM symptoms manifest clinically within 24–48 hours following chemotherapy (Cinausero et

al., 2017). Although Sonis et al., (2009) remarked that the processes of OM and GM were probably comparable, more research is still needed.

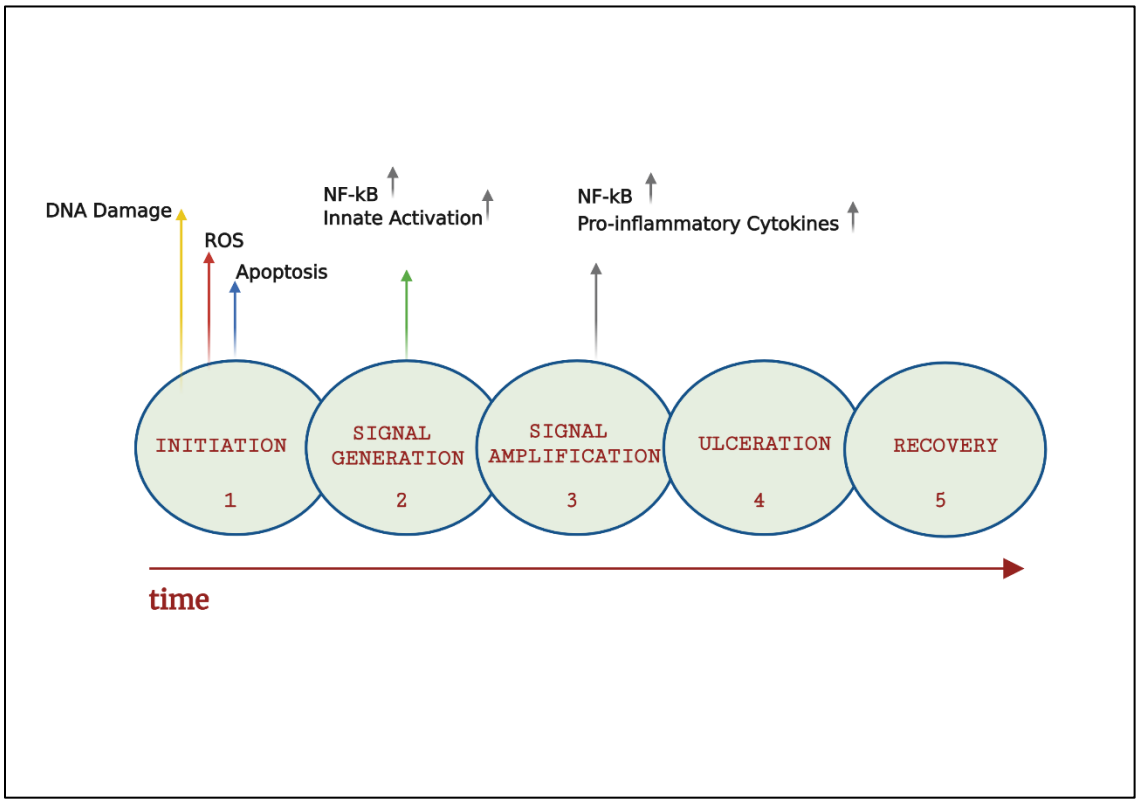
Sonis has called the process of cell death during mucositis apoptosis. There is emerging research that if apoptosis is occurring, cell death may not be limited to apoptosis but that other processes may be underway, particularly necroptosis (Choi et al., 2020). For the purpose of this thesis, I will continue to use apoptosis.

1.3. Lessons from rodent studies of mucositis

The 5-stage model of mucositis described by Sonis provides some leads into which molecular or cellular events may be targeted for intervention. Mice have been used widely in this capacity. Some examples will be provided here.

ROS generation and oxidative stress are involved in the initiation phase of CIM, leading to DNA damage and cell death (Lee et al., 2014; Li et al., 2022). Rtibi et al. (2018), using 5-FU to induce IM in rats, demonstrated decreases in multiple antioxidant biomarkers, superoxide dismutase, catalase and glutathione peroxidase, confirming the oxidative stress role in the initiation of IM. Predictably, antioxidant use has been shown to play roles in anti-inflammatory protection, decreasing injury severity *in vivo* (Nguyen et al., 2022). MS-Superoxide Dismutase, a novel SOD product, reduced 5-FU-induced IM by inhibiting oxidative stress and inflammation (Yan et, al., 2020). Reducing ROS would presumably reduce apoptosis and ultimately the inflammation that follows, but any strategy to use antioxidants needs to be proven to not also protect cancer cells.

A



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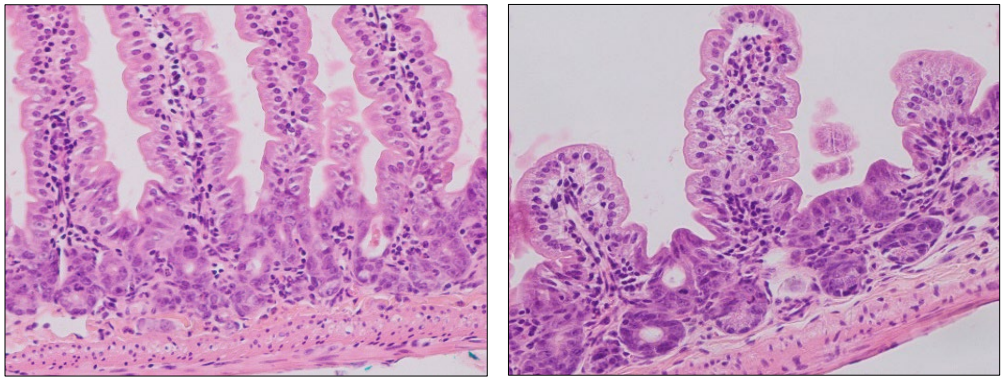

Figure 1.5 A The 5 overlapping phases of Sonis's model for mucositis development. The *Initiation phase* includes DNA damage, apoptotic cell death, ROS generation; *message generation phase* is distinguished by NF-κB activation, innate immune activation and inflammation; *signaling and amplification phase* is associated with increased NF-κB activation and increased pro-inflammatory cytokines and chemokine levels; finally, *ulceration* and *healing* phases. **B** Photomicrographs showing healthy and inflamed jejunum to illustrate the contrast in the appearance of the villi. (Photomicrographs are originals at magnification 200X.)

The severity of CIM has been linked to a number of pro-inflammatory cytokines (Guabiraba et, al., 2014). IL-1β is elevated and mediates the course of intestinal apoptosis after 5-FU administration (Wu et al., 2011a). IL-1β causes tissue damage by triggering MMPs and by enhancing apoptosis and stimulating the secondary wave of mediators (Bertolini et al., 2017; Campos et al., 2014; Sonis, 2012; Villa & Sonis, 2016). It has been demonstrated that IL-1Ra administration prohibited the destructive impact of IL-1β and decreased apoptosis in the small intestinal crypts after chemotherapy. Mice treated with a single dose of 5-FU followed by an injection of IL-1Ra were protected against intestinal apoptosis which attenuated the severity of IM, facilitated the intestinal mucosa recovery, and enhanced intestinal crypt proliferation (Wu et al., 2011a). The protection was associated with blocking the up-regulation of Bax and caspase 3 and inhibiting the downregulation of Bcl-2 and Bcl-xL (Wu et al., 2011a). Moreover, *in vitro*, IL-1Ra inhibited apoptosis and thereby increased cell viability in rat enterocyte IEC-6 cells treated with 5-FU (Wu et al., 2011a). In another study by Wu et al., (2011b), it was demonstrated that IL-1Ra treatment of mice reduced acute lethality, eliminated severe diarrhea, accelerated body weight recovery, and mice had less damage and a faster recovery of their small intestine (Wu et al., 2011b). In a related study by Xiang et, al. (2011), mice were injected intraperitoneally with rhIL-1Ra (1 mg/kg/day) once a day for 5 days followed by a single intraperitoneal injection of cyclophosphamide, a non-cell-cycle-specific cytotoxic chemotherapy agent. The pre-treatment with rhIL-1Ra protected the animals' jejunums, reduced diarrhea and body weight loss after cyclophosphamide administration. Despite these favourable outcomes in mucositis, any drug that blocks apoptosis risks reducing the efficacy of the treatment against cancer cells. The finding that IL-1 directly impacts chemotherapy induced apoptosis identifies a role separate from regulating inflammation

triggered by apoptosis but the IL-1Ra needs to be applied to animals being treated for cancer.

IL-18, a member of the IL-1 family and not included in Sonis's model, has also been implicated in mucositis. Like IL-1β, IL-18 is translated as a pro-protein requiring cleavage by the intracellular protease caspase 1 to become active (Lima-Júnior et al., 2014). Using IL-18 or caspase-1 deficient mice or IL-18 binding protein (an antagonist) protected the mice from irinotecan CIM (Lima-Júnior et al.,2014). Mice lacking caspase 1 will also lack IL-1β, risking confounding observations of the role of IL-18; however, this was compensated for by using IL-18 gene deficient mice.

IL-33, another member of the IL-1 cytokine family, has also been implicated in the mucosal damage during cancer chemotherapy (Guabiraba et, al., 2014). IL-33 and the IL-33 receptor (ST2) were both significantly elevated in the small intestine following irinotecan injections compared to PBS-treated control mice (Guabiraba et al., 2014). The authors chose 3 approaches to confirming that IL-33 played a role in IM. Mice deficient in ST2, treatment with anti-IL-33 antibody or soluble ST2 were all protected from mucositis, suggesting that IL-33/ST2 pathway blockage may represent a novel approach to improve the effectiveness of chemotherapy by limiting inflammatory mucosal damage.

The third stage of the Sonis model is the initial inflammatory response becoming amplified by proinflammatory cytokines, in particular TNF (Huang et al., 2022). TNF is highly pleiotropic, stimulating other cytokine expression including IL-1β and IL-6, and directly impacts the survival of other cells. Therefore, it should be no surprise that TNF inhibitors were discovered to lessen the severity of OM in a hamster model of 5-FU-induced CIM (Huang et al., 2022).

1.4. The complement system

Complement, a potent element of innate immunity (Dunkelberger & Song, 2010; Klos et al., 2009; Lambris et al., 2010; Liszewski et al., 1991), is a network of more than 20 soluble zymogens and membrane-bound proteins, multiple split effector molecules, cellular receptors and regulatory molecules (Klos et al., 2013; Lublin & Atkinson, 1989). Intestinal epithelial cells, infiltrating macrophages, neutrophils, T cells and perfusing blood (bleeding in the ulcerated regions) are all potential sources of complement (Andoh et al., 1993; Cortes et al., 2012). Complement may be activated and amplified after microbial invasion or tissue damage through three different biochemical pathways: the ***classical***, ***alternative***, and ***lectin*** pathways (Dunkelberger & Song 2010; Ricklin & Lambris, 2013; Riihilä et al., 2019). During activation all 3 routes progress through a series of proteolytic cascades (Ehrnthaller et al., 2011; Jain et al., 2014; Klos et al., 2013; Lambris et al., 2010). Each pathway requires unique initiation events but all converge at the C3 convertase stage, amplifying cleavage of C3 then cleavage of C5 and ultimately formation of the membrane attack complex (MAC) (Lambris et al., 2010; Sünderhauf et al., 2017; Zhang et al., 2018b). The ***classical pathway (CP)*** is regarded as an antibody-dependent activation pathway. It is activated through antigen-bound IgM and IgG, which bind C1q becoming a C1 (C1qr2s2) complex, a pattern recognition molecule. C1q binds to the Fc-region of the antibody which activates C1r and cleaves C1s. Then C4b binds to the microbe surface and cleaves C2 into C2b and C2a. Association of C2b (formerly called C2a) with C4b form the classical C3 convertase (C4b2b) (Lambris et al., 2010). This C3 convertase in turns splits C3 into C3a and C3b; C3a (an anaphylatoxin) diffuses away while C3b acts as an opsonin by interacting with receptors CR3 and CR4 on phagocytes or associates with the C3 convertase to form a CP C5 convertase (C4b2b3b), which in turns cleaves C5 into C5a (an anaphylatoxin) and

C5b (Ricklin & Lambris, 2013). C5b then initiates the terminal phase of complement activation to form the MAC ($C5b6789_n$) (Figure 1.6) (Dunkelberger & Song, 2010; Esser, 1994; Riihilä et al., 2019). The ***Alternative pathway (AP)*** is an antibody independent activation pathway. Activation begins with the spontaneous hydrolysis of C3 to $C3(H_2O)$ or the pathway can be activated by various microorganisms (Gasque ,2004; Rosas et al., 2002; Muller-Eberhard, 1988). $C3(H_2O)$ binds factor B (fB), which then is cleaved by factor D (fD) to form the AP C3 convertase ($C3b(H_2OBb)$ (Bexborn et al., 2008). This C3 convertase splits C3 producing more C3a and C3b. C3b, again, is an opsonin that also generates a new C3 convertase (C3bBb) with further fB and fD (Thurman & Holers, 2006), or binds the C3 convertase to make a C5 convertase and then MAC (Figure 1.6). However, the AP C3 convertase and possibly the C5 convertase are relatively unstable and therefore are stabilized by properdin (Di Sabatino et al., 2003; Harboe & Mollnes, 2008). ***Lectin pathway (LP)*** activation is triggered by binding of mannose binding lectin (MBL) or other lectins (ficolins 1,2 and 3 and collectin-11), to carbohydrate moieties on microbial surfaces (Endo et al., 2015; Hansen et al., 2010). Activation is triggered by recognizing and binding of MBL to N-acetylglucosamine and mannose moieties on microbial surfaces (Endo et al., 2015; Hansen et al., 2010).This binding causes MBL-associated serine proteases (MASP-1, 2 and 3) activation and recruitment to form a complex with a similar activity to the C1complex of the CP. MASP-2 cleaves C2 and C4 forming the C3 convertase identical to the CP (Lambris et al., 2010; Reid & Turner, 1994; Vorup-Jensen et al., 2000) (Figure 1.6). Independent of these 3 pathways of activation, C3a and C5a generation can also be achieved through the "extrinsic protease pathway" by proteases including cathepsin L and D directly cleaving C3 and C5 respectively, or coagulation factors thrombin, plasmin, factor Xa and Factor XIa (Amara et al., 2008; Amara et al., 2010; Ward et al., 2006).

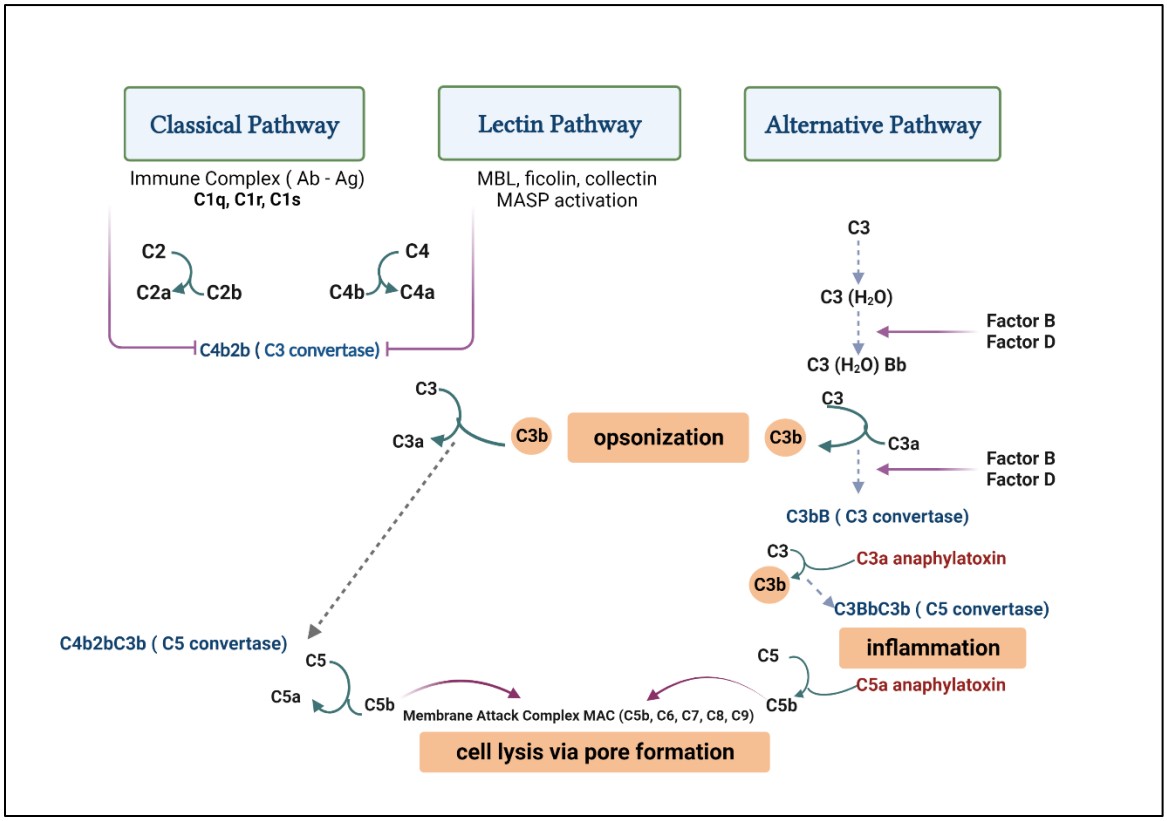

Figure 1.6 Schematic of the pathways of complement activation and functions. The three pathways converge at the C3 convertase stage (C3bBb for the alternative pathway and C4b2b for the classical and lectin pathways*)*, heightened cleavage of C3 generates copious amounts of C3a and C3b. C3b association with the C3 convertase results in a C5 convertase, which cleaves C5 into C5a and C5b. C3b is an opsonin, C3a and C5a are the anaphylatoxins, and C5b initiates membrane attack complex formation.

Complement carries out three main functions;1) opsonization, 2) direct lysis of target cells through MAC, and 3) inflammation through the anaphylatoxins (Dunkelberger & Song, 2010; Lambris et al., 2010). Complement molecules contribute to the elimination of apoptotic cells and microbes by acting as opsonins, facilitating phagocytosis by professional phagocytes (Brekke et al., 2007; Choteau et al., 2016; Gasque, 2004; Hajishengallis et al., 2017; Klos et al., 2009; Mevorach et al., 1998). Apoptotic cell clearance by DCs and macrophages is directly facilitated by the opsonization of apoptotic cells with C1q, MBL, IgG, IgM, C4b, C3b, iC3b or C3d, which enable phagocytes to recognize the targeted cell and bind forming a bridge between the cells (Kang et al., 2012; Krysko et al., 2006; Nauta et al., 2004; Trouw et al., 2008; Vandivier et al., 2002). Complement components attach to apoptotic cells directly or attach by other plasma proteins such as IgG, IgM, C Reactive Protein (CRP) and histidine rich protein (Trouw et al., 2008; Fishelson et al., 2001). Apoptotic cells may also express surface C3 fragments and are engulfed through C3 receptors (CR1, CR3 and CR4) on phagocytes (Choteau et al., 2016; Morelli et al., 2003; Verbovetski et al., 2002). CD91 is a receptor on phagocytes for C1q bound to IgM/IgG on apoptotic cells (Fishelson et al., 2001). CD91 also binds to MBL found on apoptotic cells (Ciurana et al., 2004; Ogden et al., 2001; Quartier et al., 2005; Trouw et al., 2008 ; Zwart et al., 2004). MBL and L and H ficolins are important molecules in the LP (Choteau et al., 2016; Kuraya et al., 2005) but are also able to mediate phagocytosis. Apoptotic cells are removed when C1q and MBL bind to surface of CD91 and calreticulin (cC1q receptor) causing initiation of micropinocytosis and uptake of apoptotic cells (Ghiran et al., 2000; Ogden et al., 2001; Sim et al., 1998; Vandivier et al., 2002). Binding and activation of complement molecules take place in the late phase of apoptotic cells (Trouw et al., 2008).

Many studies have used mice deficient in complement molecules in models of intestinal disease. However, there is limited evidence in the literature in human and animal models for a direct link between complement and CIM pathogenesis. In an earlier study using mice deficient in properdin (intended to quench the alternative pathway), Jain et al, (2017) showed that complement becomes activated during 5-FU-induced CIM in mice and that properdin deficient mice were significantly protected from mucositis through a complement activation independent mechanism. They also showed that a C5aR antagonist was without benefit in mice, ruling out a role for C5a in IM (Jain et al., 2017). OM was not assessed in the study.

1.5. Research hypothesis and objectives

There is still a significant knowledge gap regarding the understanding of molecular pathways underlying the harm brought on by chemotherapy that leads to OM and/or IM. Despite the well characterized contribution of complement activation to inflammation, including at mucosal sites, there are no studies confirming a role for complement in the mechanisms of mucositis. The hypothesis raised is that the complement system significantly contributes to the inflammatory process in mucosal sites (intestine and oral cavity) triggered by chemotherapy drugs. The objectives were to assess IM and OM due to 5-FU or MTX, chemotherapy drugs used in humans that lead to a high incidence of mucositis, in mice lacking MBL pathway or C3.

CHAPTER 2: MATERIALS AND METHODS

2.1. Mice and ethical considerations

C57BL/6 (used as MBL^{WT} and $C3^{WT}$, Jackson Laboratories, Bar Harbor, ME), B6;129S4-C3tm1Crr/J ($C3^{KO}$, Jackson Laboratories, JAX stock #029661, Wessels et al., 1995) and B6.129S4-Mbl1tm1Kata Mbl2tm1Kata/J (MBL^{KO}, JAX stock #006122, Shi et al., 2004) mice were housed and bred in Specific Pathogen Free (SPF) conditions in the IWK Health Centre in vivo lab. For the MBL experiment, 2 mice of each genotype were co-housed in a single cage for 2 weeks prior to drug injections. For the C3 experiment, $C3^{WT}$ were bred with $C3^{KO}$ to produce heterozygous mice ($C3^{HZ}$). The $C3^{HZ}$ F1 siblings were bred and the F2 offspring used in experiments. The F2 offspring, at weaning, were divided into cages by sex without knowledge of the genotypes, the maximum number of mice in any single cage reaching 4. Mice were used in experiments when 7-10 weeks of age. Each mouse had a unique ear punch pattern, with the tissue frozen at -80°C until used for identifying the genotype. Mice were maintained in plastic cages and had free access to chow pelleted food and water *ad libitum* and were housed on woodchip bedding on a 12-h dark/light cycle in a temperature and humidity-controlled room. The experiments were undertaken with the approval of the University Committee on Laboratory Animals, Dalhousie University, who in turn adjudicate the standards of the Canadian Council of Animal Care. One noteworthy early endpoint was that any animal losing greater than 15% of their body weight compared to the animal's weight before treatment, was immediately euthanized.

2.2. Animal handling, induction of mucositis and clinical health measures

The common regimen for eliciting mucositis was by administering 75 mg/kg of 5-FU or MTX (Sigma-Aldrich, stock prepared in DMSO then diluted to deliver 200 µl in

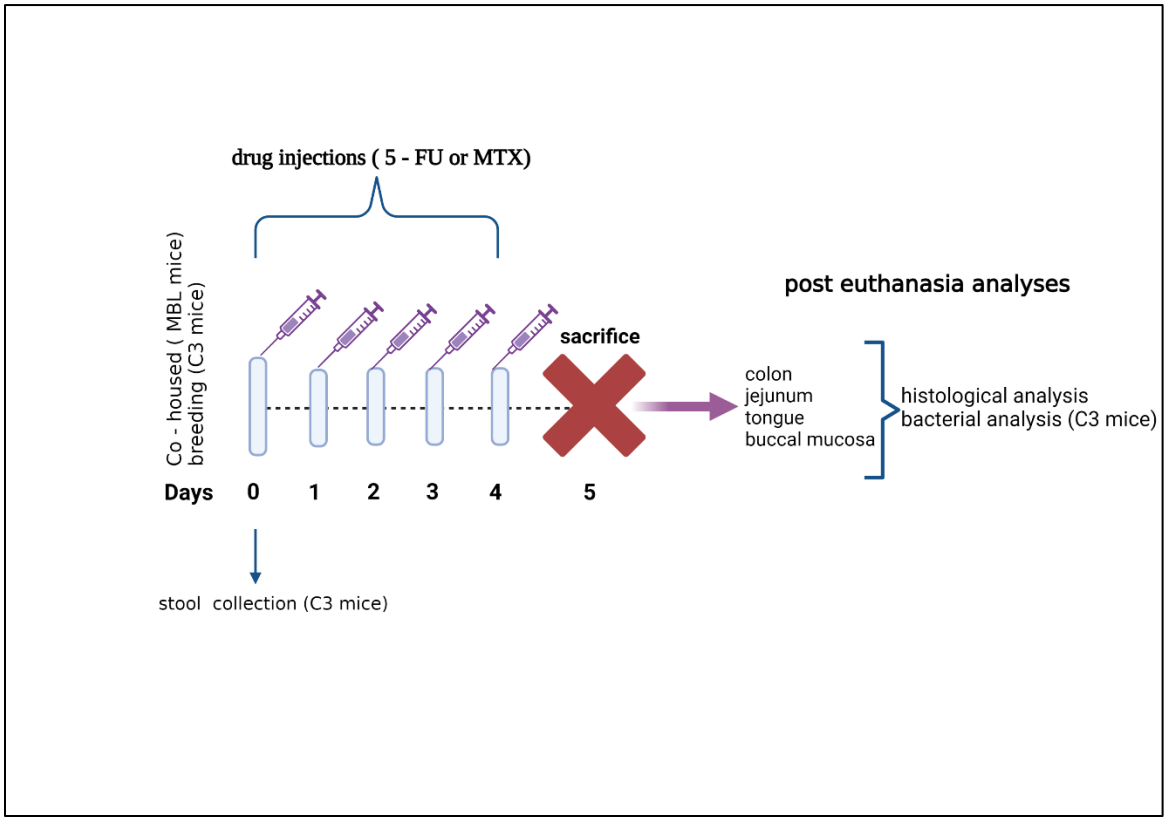

Figure 2.1 Schematic of the study design. MBL^{WT}, MBL^{KO}, $C3^{WT}$, $C3^{KO}$ and $C3^{HZ}$ mice were treated with drug (75 mg/kg 5-FU or MTX) in 5 intraperitoneal injections, then 24 hours after the final injection mice were euthanized. Body weight was tracked daily, stool samples were collected prior to the first injection for bacterial analysis. Colon, jejunum, tongue, and cheek mucosa were extracted for histopathological studies.

phosphate-buffered saline (PBS) intraperitoneally once daily beginning on day 0 (total of 5 injections), and the mice killed and samples collected 24 hours later (Figure 2.1). Any exceptions to this regimen are identified in the results. Each animals' weight was tracked daily. In the $C3^{KO}$ experiment stool specimens of mice were collected before the first drug injection. To collect fresh stool, each mouse was placed into a clean cage lacking bedding until it defecated or for 15 minutes (in which case it may be returned to the clean cage later). The stool was collected into a microfuge tube and kept on ice until all the specimens were collected. The stool was observed for consistency and whether blood was detectable (Hemoccult strips, Beckman Coulter, Brea, CA). The consistency was categorized as normal; soft with no blood; normal with occult blood; soft with occult blood; soft with visible blood and diarrhea. Stool used for bacterial analyses was stored at -80°C.

The genotype of C3 mice was confirmed by using Polymerase Chain Reaction (PCR) protocols available at Jackson Laboratories, and were conducted by Katerina Allan, technician. Reagents for genotyping included the KAPA HotStart Genotyping Kit (SKU# KK7351, Millipore Sigma, Oakville, ON). The ear tissue from each mouse was added to a master mix including 88µl of distilled water, 2µl of 1U/µl KAPA Express Extract Enzyme and 10µl of 10X KAPA Express Extraction Buffer (Sigma-Aldrich, Oakville, ON). The cells were lysed by using a pre-set program in a thermocycler. The cycle ran at 75°C for a period of 10 minutes followed by a 5 minute incubation at 95°C. DNA extracted from the lysis was added to 12.5µl of 2X KAPA FAST (HotStart) genotyping mixed with dye, 1.25µl of 10µM forward primer (5′ ATCTTGAGTGCACCAAGCC3′), 1.25µl of 10µM reverse primer (5′GGTTGCAGCAGTCTATGAAGG3′), 1.25µl of 10µM Neo primer (5′GCCAGAGGCCACTTGTGTAG3′) and 7.75µl of distilled water to a final volume of 25µl. The thermocycler was set up as follows: (initial denaturation) 3 minutes at 95°C,

(denaturation) 15 seconds at 95°C, (annealing) 15 seconds at 55°C, (extension) 15 seconds at 72℃, and (final extension) 1 minute at 72℃. The thermocycler ran for a total of 40 cycles. Products of PCR and a DNA 100 base pair ladder were run on an ethidium bromide (0.5μg/ml) stained 1.5% agarose gel in Tris-acetate-EDTA buffer pH 8.3.

2.3. Necropsy sample collection and histopathology

Mice were deeply anesthetized by 5% isoflurane inhalation until the pedal reflex was lost, then sacrificed by cervical dislocation. The heads of mice were cut from the torso and immediately fixed in 10% buffered formalin, for a minimum of 5 days, after which the formalin was replaced with 70% ethanol in preparation for dissection of the tongue and cheek mucosa. The jejunum and colon were excised, flushed with cold PBS to remove feces, then held on ice throughout processing. Both intestines were opened longitudinally and laid flat then divided into 2 lengthwise strips. A Swiss roll was prepared from one strip of colon for histology. A strip of the jejunum was cut into smaller pieces and added to the same cassette as the rolled colon, then both tissues were fixed in 10% buffered formalin for a maximum of 24 hours when the formalin was replaced with 70% ethanol. The intestines were processed by the Pathology and Laboratory Medicine Service, IWK Health Centre, Halifax. Briefly, fixed samples were dehydrated in a series of graded alcohols (50%, 70% and 95%) for 30 minutes each, then embedded in paraffin wax, cut into 4μm sections, mounted onto silane coated slides, dewaxed, rehydrated, and stained using hematoxylin and eosin (H&E). For tongue and cheek mucosa samples, skin was removed from the sample to facilitate processing. The buccal mucosa was placed on edge, and longitudinal sections of the tongue placed *en face*, and both held in place using agar. The tissues were cut into 5μm sections and stained with H&E at the Dalhousie Oral Pathology Service, Faculty of Dentistry. Histological sections were photographed using a LEICA

DM1000 light microscope equipped with a LEICA MC170 HD camera.

Small intestinal sections were evaluated for villus shortening, epithelial damage, crypt damage, apoptosis, cellular inflammatory infiltration and ulceration (Table 2.1), as previously reported by Jain et al., 2017**.** Oral sections (tongue and cheek mucosa) were evaluated for epithelial hyperplasia, mitosis, ulceration, and cell infiltration.

To report the number of goblet cells in the colon specimens, I counted the total number of epithelial cells in an entire crypt, then the number of goblet cells in the same crypt, for 10 crypts in sections for each mouse. The average number of goblet cells in the 10 crypt sample was calculated and the data reported as (# goblet cells/ #epithelial cells - #goblet cells).

2.4. Characterization of stool bacteria

The fecal samples were thawed, suspended in PBS then centrifuged at 14,000 ×g, and the pellets resuspended in 100mM Tris-HCl (pH 9.0) containing 40mM EDTA and 1% sodium dodecyl sulfate. The suspension was mixed with buffer-saturated phenol and 0.1mm glass beads and shaken at 800 ×g for 10 seconds. Following centrifugation at 14,000 ×g (5 minutes), the supernatant was collected, extracted with phenol-chloroform and DNA precipitated with isopropanol. The resulting DNA pellet was washed with 70% ethanol, dried and dissolved in 10mM Tris-HCl (pH 8.0) with 1mM EDTA. The composition of the intestinal bacterial community was analyzed based on sequencing the V3-V4 region of 16S rRNA genes and bioinformatics analyses was conducted at the Integrated Microbiome Resource, Dalhousie University (http://imr.bio/, Comeau et al*.,* 2017). Sequence files were visualized using the Quantitative Insights Into Microbial Ecology 2 (https://view.qiime2.org) Emperor Ordination: unweighted UniFrac and STAMP (Parks et al., 2014).

Table 2.1 Histopathological scoring of chemotherapy-induced mucositis.

Score	Villous height	Apoptosis	Epithelium	Inflammation	Crypt loss
0	normal	normal	normal	normal	normal
1	short	focal	reactive	mild	Limited
2	blunt	diffuse	eroded	moderate	Extensive
3	severe	severe	ulcerated	severe	severe

CHAPTER 3: RESULTS

3.1 Mannose Binding Lectin deficiency and 5-fluorouracil induced mucositis

3.1.1 Clinical illness symptoms and pathological phenotypes in MBL^{KO} mice

It has been demonstrated that 5-FU administration (Chang et al. 2012; Jain et al. 2017) and other chemotherapy drugs (Al-Dasooqi et al*.,* 2010; Logan et al*.,* 2008; Xiang et al., 2011), result in clear histopathological mucositis, evident through gross architectural disturbances occurring in GIT tissues, most notably in the jejunum and colon of mice. Upon administration of 5-FU for 5 days as a single daily dose intraperitoneally, MBL^{KO} mice that were co-housed with MBL^{WT} mice, responded similarly, including losing weight at the same rate (Figure 3.1). Moreover, mouse stool consistency late in the regimen was typically soft pellets in both mouse genotypes, with blood observed in 1 mouse of each genotype. The occurrence of soft stools is suggestive of inflammation and blood is indicative of ulcers.

3.1.2. Lack of Mannose Binding Lectin does not protect the jejunum from mucositis

Further to the objective of this study, to evaluate whether MBL and the lectin complement pathway contribute to mucositis, I compared the histology of jejunum samples of the 5-FU-treated MBL^{KO} and MBL^{WT} mice. First, untreated MBL^{KO} mouse jejunum was examined for any sign of inflammation. Figure 3.2 shows MBL^{KO} mice have uninflamed intestines. The regimen I used elicits clear evidence of a variety of histopathological manifestations in the jejunum of mice treated with 5-FU. As shown in Figure 3.2, MBL^{KO} and MBL^{WT} mice showed comparable histopathological results consistent with IM. Histopathological features in the jejunum included extensive epithelial damage, villous atrophy, nuclear changes in size and shape, the destruction of crypts including crypt loss, crypt atrophy, small spaces between crypts, focal ulceration, a moderate leukocyte

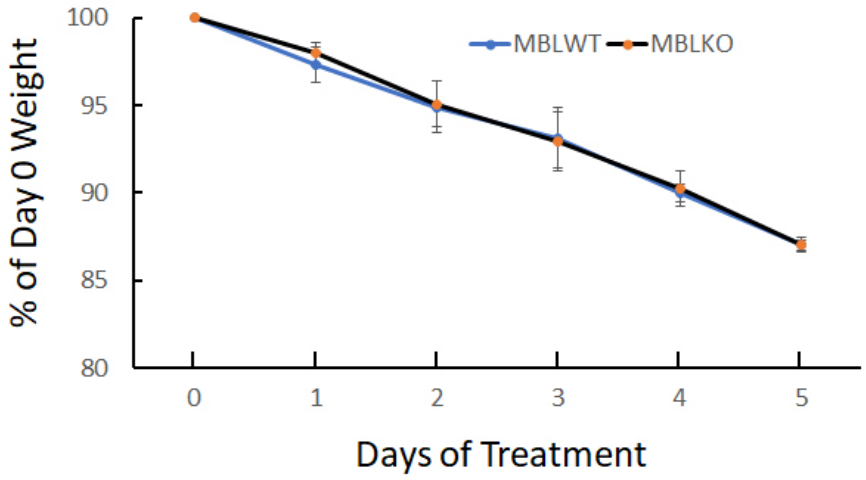

Figure 3.1 5-fluorouracil-induced clinical illness symptoms and pathological phenotypes in Mannose Binding Lectin knockout and wild type mice. MBL^{WT} and MBL^{KO} mice received a daily injection of 75 mg/kg (5 intraperitoneal injections), then 24 hours after the final injection mice were euthanized (n= 4/each group). Mice responded similarly, losing weight at the same rate. Body weight was recorded daily. The early end point in weight loss is ≥15%.

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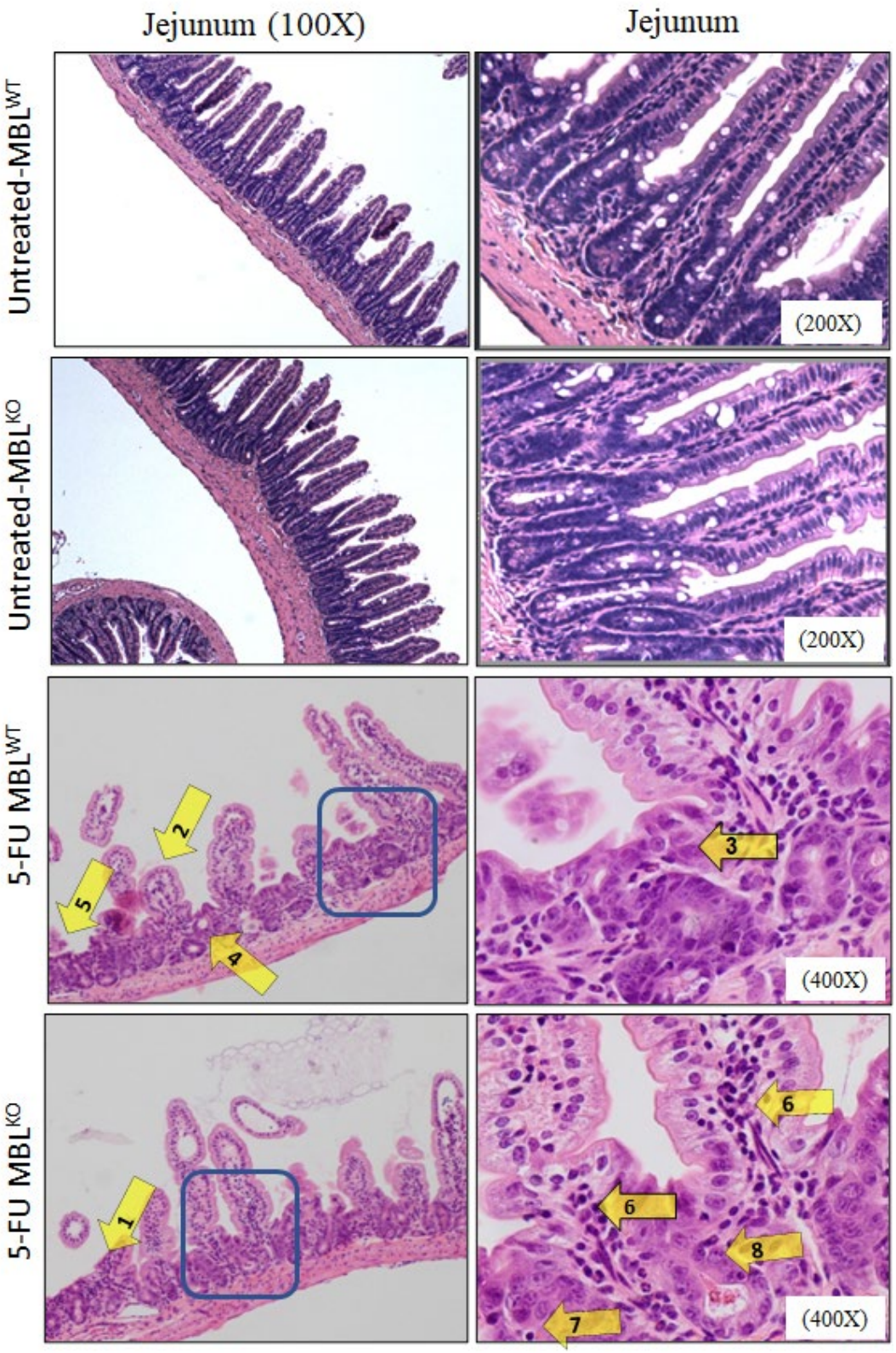

Jejunum (100X)
Jejunum
Untreated-MBL^WT
(200X)
Untreated-MBL^KO
(200X)
5-FU MBL^WT
1
2
3
4
5
(400X)
5-FU MBL^KO
6
6
7
8
(400X)

B

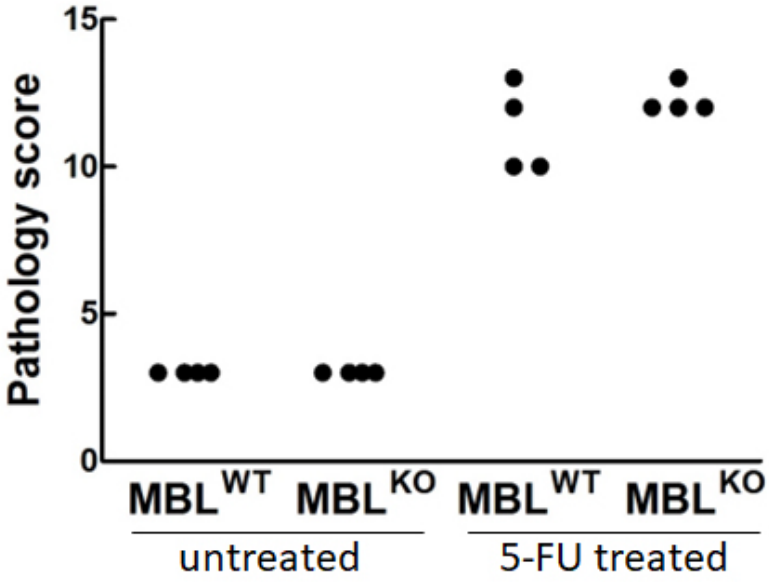


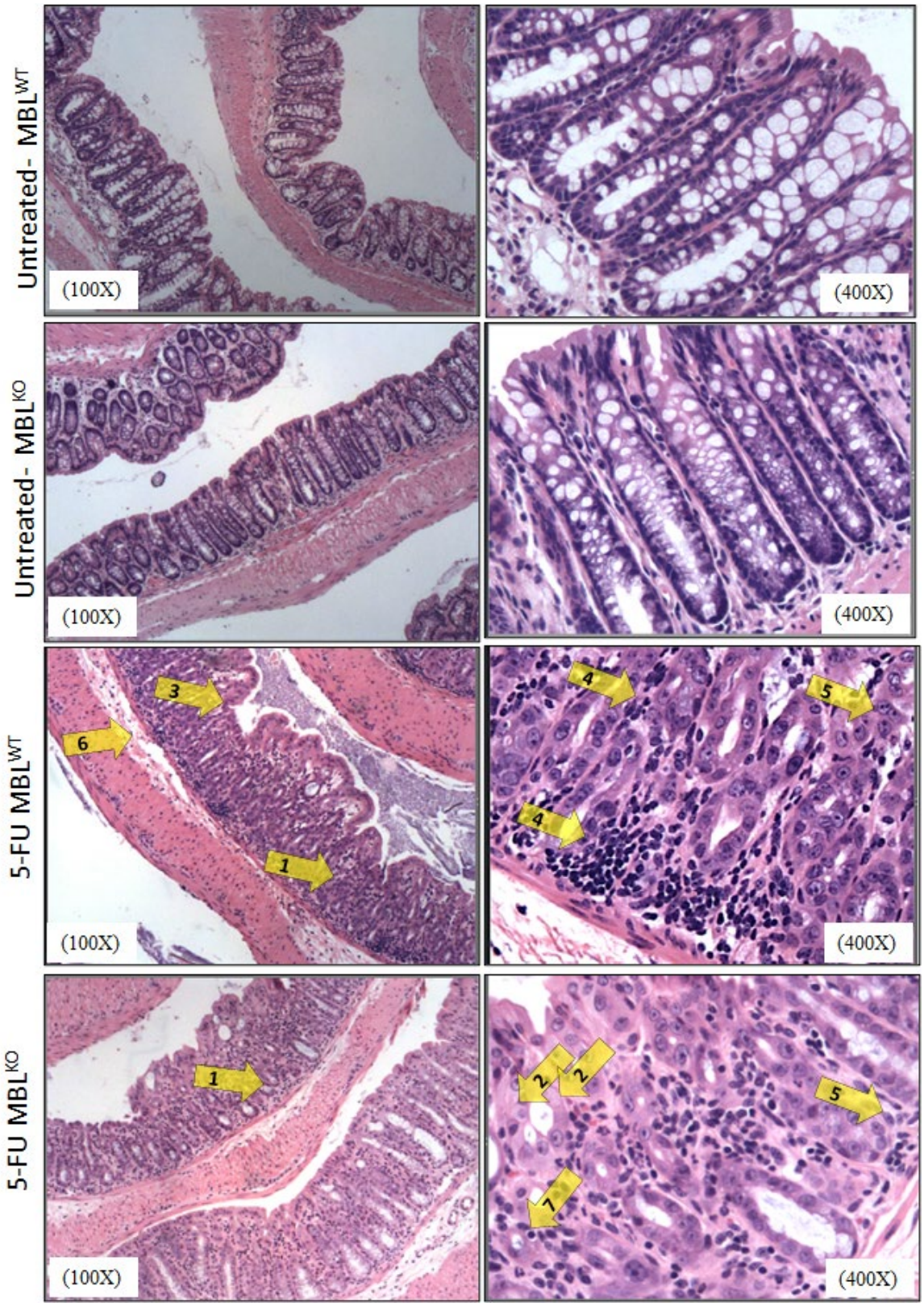
Figure 3.2 Lack of Mannose Binding Lectin does not protect the jejunum from mucositis. **A** Representative photomicrograph of the jejunum stained with H&E, prepared from 5-FU-untreated and treated mice of MBL^{KO} and MBL^{WT}. Jejunal samples of 5-FU-treated mice demonstrated epithelial damage (arrow 1) villus shortening (2), nuclear changes in shape and size (3), crypt destruction with obvious crypt loss, crypt atrophy, and small spaces between crypts (4) focal ulceration (5), moderate leukocytes infiltration in both the lamina propria and the crypts (6), apoptosis (7), mitosis (8). Boxes on the left panels indicate the section viewed using higher magnification in the panels on the right. Sections were examined using a LEICA DM1000 light microscope equipped with a LEICA MC170 HD camera. **B** Pathology scores, the sum of the 5 criteria in Table 2.2. Each dot represents a single mouse.

infiltration in both the lamina propria and the crypts, and obvious mitosis, apoptosis, and hypertrophy occurred in the smooth muscle layer. The conclusion is that MBL deficiency does not protect the jejunum from CIM.

3.1.3. Lack of Mannose Binding Lectin does not protect the colon from mucositis

The small intestine is the organ commonly reported as inflamed by chemotherapeutic drug injections in mice, whether the animal's colon becomes inflamed is less often reported. Untreated MBL^{KO} mouse colons were examined for signs of inflammation. Figure 3.3A shows MBL^{KO} mice have uninflamed intestines. The colon in MBL^{KO} and MBL^{WT} mice manifested various histopathological features as shown in Figure 3.3A. Others have reported the number of goblet cells in the colon appeared to be reduced due to discharging mucins in rats inflamed with mucositis (Stringer et al., 2009; Thorpe et al., 2020). From the histology, I also detected an apparent reduction in goblet cell numbers (Figure 3.3B), followed by epithelial damage with pleomorphic nuclear changes in size and shape and with irregular orientation, and enlargement of nuclei in much of the epithelium. Focal ulceration and a moderate leukocyte infiltration in both the lamina propria and the crypts (cryptitis) are observable. Furthermore, there is evidence of mitosis, edema, vascular dilatation in the submucosa and apoptosis of epithelial cells is hallmarked by chromatin condensation. Features of intestinal pathology were similar in both genotypes of mice; epithelial damage, villus shortening, leukocyte infiltration, and crypt damage including cryptitis. The clinical findings, taken together with the histopathology observed in the colon and jejunum, lead to the conclusion that the absolute lack of MBL does not protect mice from CIM under the conditions of our regimen. MBL is not involved in mucositis development. Consequently, the LP triggered by MBL is not critical to CIM.

A


Untreated- MBL^WT
(100X)
(400X)
Untreated- MBL^KO
(100X)
(400X)
5-FU MBL^WT
3
6
1
(100X)
4
5
4
(400X)
5-FU MBL^KO
1
(100X)
2
2
5
7
(400X)

B

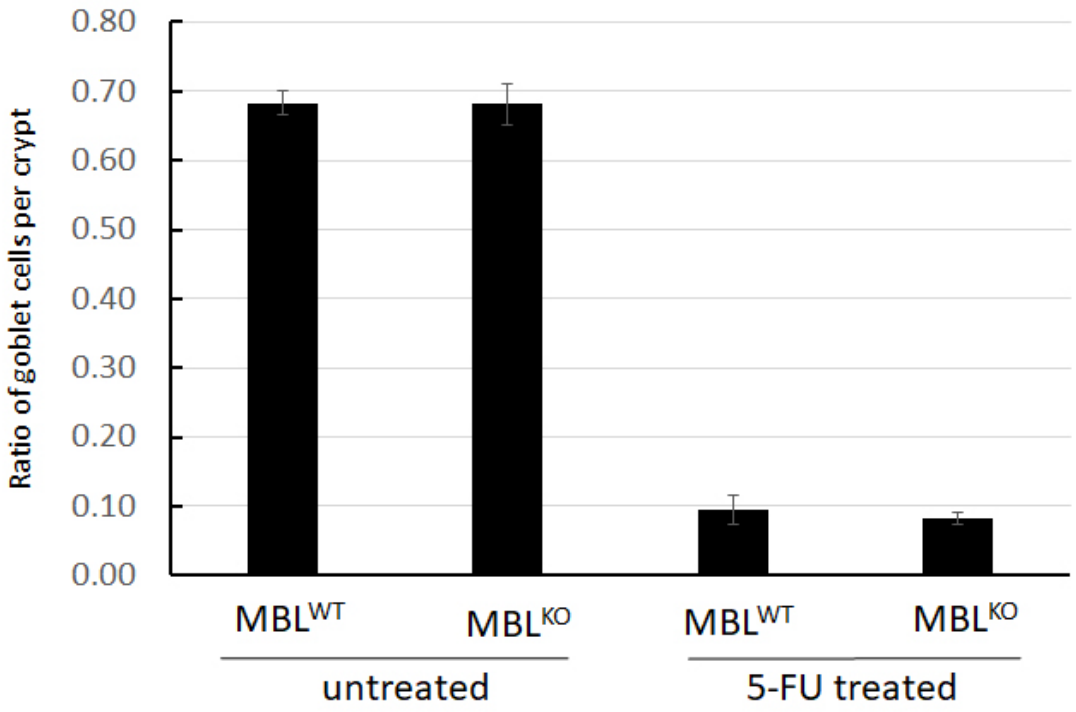


Figure 3.3 Lack of Mannose Binding Lectin does not protect the colon from mucositis. **A** Representative photomicrographs of the colon prepared from untreated and 75mg/kg 5-FU-treated MBL^{KO} and MBL^{WT} mice. Colon samples showed reduction in the number of goblet cells (arrow 1), epithelial damage with pleomorphic nuclear changes in size and shape, irregular orientation, and enlargement of nucleus (2), focal ulceration (3) moderate leukocyte infiltration in the lamina propria and crypts (cryptitis) (4), mitosis (5), edema and vascular dilation (6), and apoptosis (7). Sections were examined using a LEICA DM1000 light microscope equipped with a LEICA MC170 HD camera. **B** The number of goblet cells as a percentage of all other epithelial cells in the crypts shows the reduction following 5-FU treatment. n=4 ± standard deviation.

3.1.4. Mannose Binding Lectin deficient mice do not develop oral mucositis

The obvious clinical manifestation of CIM in humans occurs in the oral cavity. On the other hand, eliciting OM has been challenging in rodents, with most models requiring a physical injury combined with the drug administration. Whether the oral mucosa of MBL^{KO} mice is healthy has not been reported. Figure 3.4 shows that the strain is healthy. Since the MBL^{KO} had never been reported by others to be more or less susceptible to OM, combined with the finding that the mice experience IM, I examined our mice for OM. Neither MBL^{WT} nor MBL^{KO} mice exhibited OM after our daily injection regimen. Layers of both buccal mucosa and ventral tongue were intact and devoid of signs of inflammation (ulceration, epithelial hyperplasia, increased mitoses and an inflammatory infiltrate in the lamina propria) (Figure 3.4). I concluded that although MBL^{KO} and MBL^{WT} mice treated for 5 days with 5-FU experience IM, they are not susceptible to chemotherapy induced OM.

3.2. C3 deficiency and 5-fluorouracil induced mucositis

3.2.1. $C3^{KO}$ mouse health and breeding outcomes

To date, despite complement becoming activated during the 5-FU treatment regimen, neither the alternative pathway or C5a (Jain et al., 2017) nor MBL 1 or 2 (representative of the lectin pathway) contribute to the inflammation. Split C3 molecules are central to all the complement pathways and are directly inflammatory through opsonization and the anaphylatoxin properties of C3a. Therefore, mice deficient in C3 will lack all the split C3 molecules and presumably lack split C5 molecules and the MAC, notwithstanding that C5 can be cleaved by enzymes of the clotting cascade (Amara et al., 2008; Amara et al., 2010; Ward et al., 2006). Arguably, the most effective way to extinguish the complement system to test the hypothesis is to use C3 deficient mice.

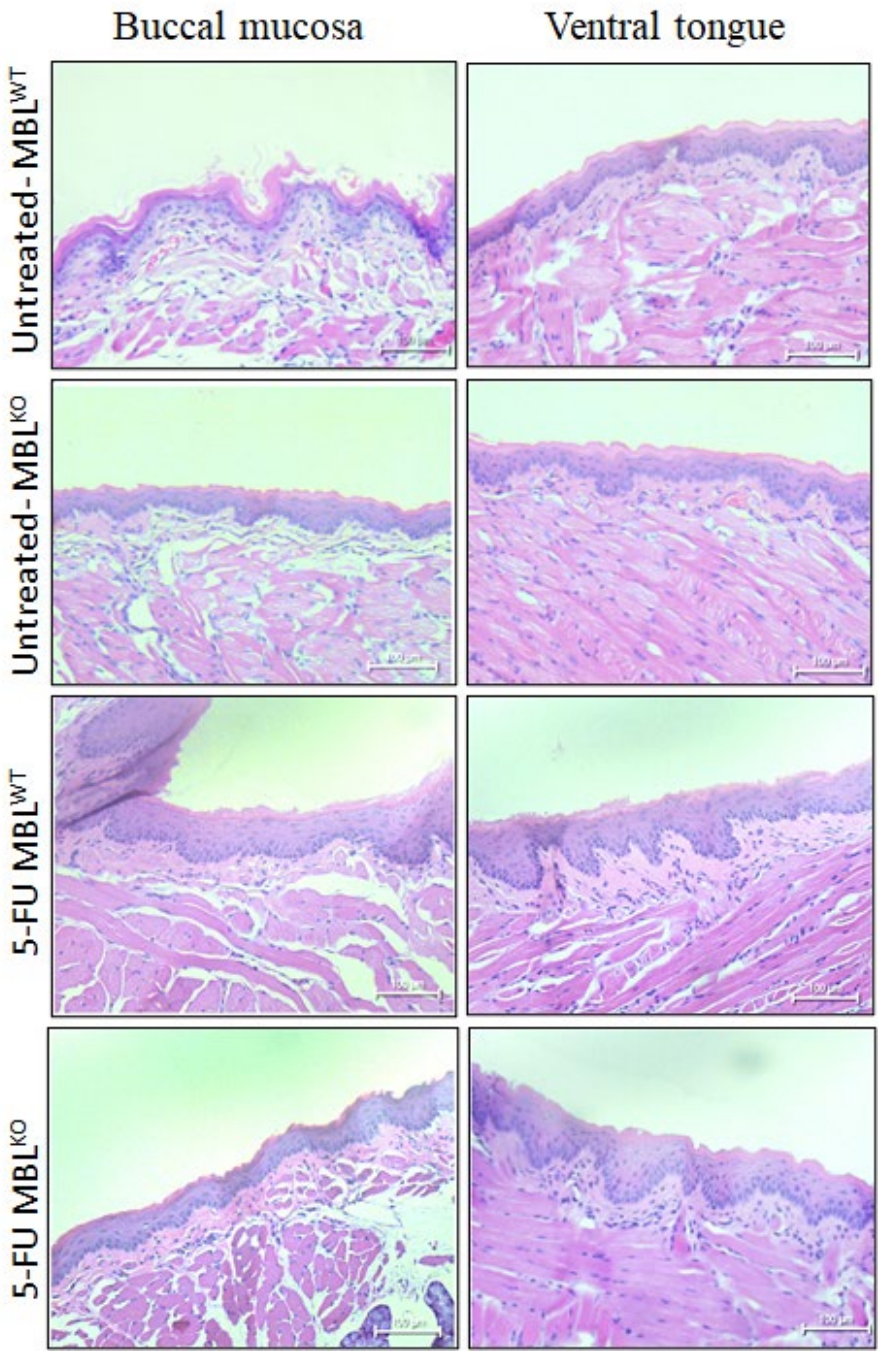


Figure 3.4 Mannose Binding Lectin deficient mice do not develop oral mucositis. Representative photomicrographs stained with H&E showing the buccal mucosa (left panels) and ventral tongue (right panels) of untreated MBL^{WT} and MBL^{KO}, and MBL^{WT} and MBL^{KO} mice treated with 5- FU (n=4/group). The keratinized stratified squamous epithelium and skeletal muscle layers are intact. Sections were examined using a LEICA DM1000 light microscope equipped with a LEICA MC170 HD camera. All images are 200X magnification.

Raised in SPF conditions, $C3^{KO}$ mice on the C57BL/6 genetic background are healthy and fertile and adults' weights are not significantly different from $C3^{WT}$ mice. $C3^{KO}$ mice showed no overt signs of illness nor spontaneous or constitutive inflammation in their intestines. Unlike MBL for which there are 2 genes, C3 is present in a single copy and therefore the $C3^{KO}$ strain is more conducive to breeding as a means of sharing their gut microbiomes. I undertook a breeding regimen that would, 1) result in the offspring from $C3^{HZ}$ breeding pairs sharing cages, and 2) examine the response of heterozygous mice, which is not commonly reported. Not knowing whether the mouse C3 alleles are co-dominant, the response of $C3^{HZ}$ mice is unpredictable and worth examining. Working with the F2 litters had another advantage, the genotypes of the mice would be unknown at the time of the drug injections. The breeding strategy to derive $C3^{KO}$ mice is detailed in Figure 3.5. Two F2 litters totaling 14 pups were born, one litter each from 2 $C3^{HZ}$ breeding pairs. The predicted ratio of each genotype among the F2 offspring is 2 $C3^{HZ}$: 1 $C3^{WT}$: 1 $C3^{KO}$. I obtained 4 $C3^{HZ}$ (3 females, 1 male), 6 $C3^{WT}$ (3 females, 3 males), and 4 $C3^{KO}$ (2 females, 2 males). Of the animals used in the study, 57% were female and 42% were male. Separating mice from each litter by sex only resulted in $C3^{KO}$ mice sharing a cage with $C3^{WT}$ and $C3^{HZ}$ littermates; however, due to the low number of $C3^{KO}$, there were $C3^{WT}$ and $C3^{HZ}$ mice that did not share a cage with $C3^{KO}$.

3.2.2. Gut bacteria from $C3^{KO}$ mice bred from $C3^{HZ}$ parents become more similar to bacteria from $C3^{WT}$ mice

The GIT harbors a great array and numbers of microbes, the gut microbiome. Recent research on the microbiome, particularly bacteria**,** has revealed multiple biological and immunological impacts including on host metabolism, the metabolism of medications and on immunological maturity and responses (Kim et al., 2020).

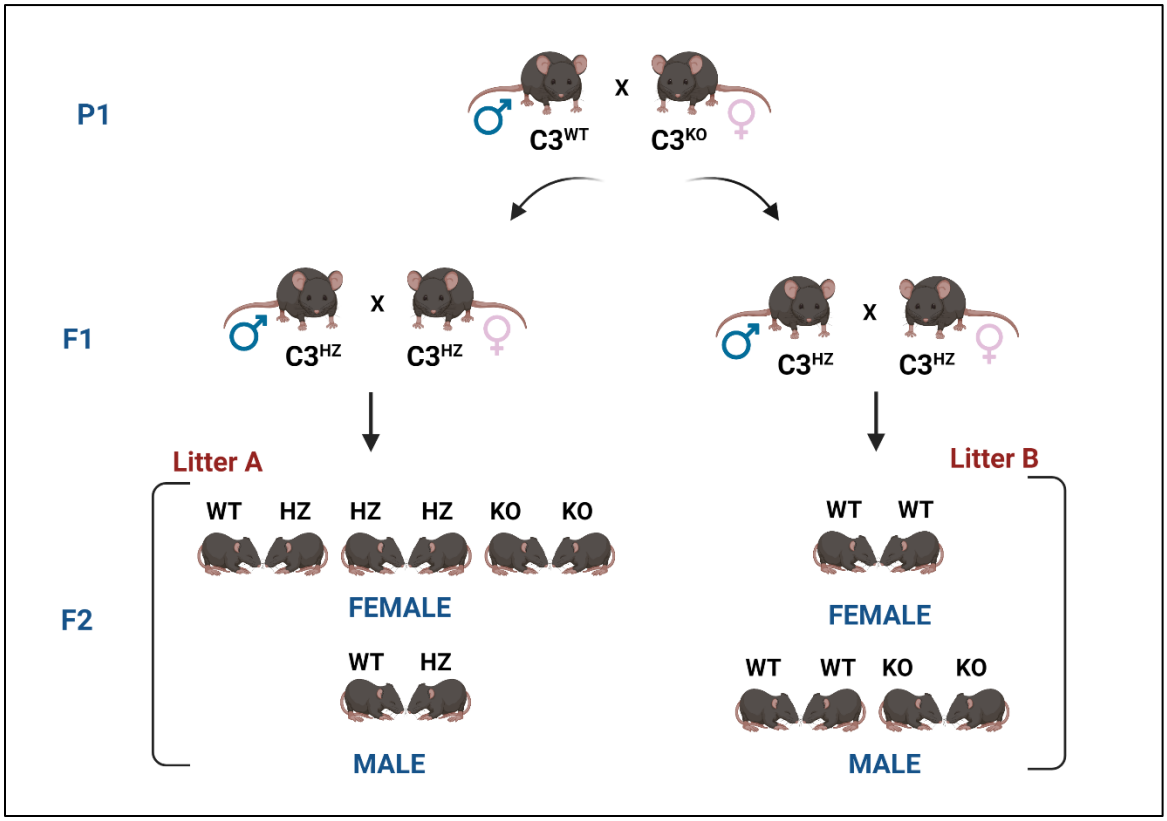
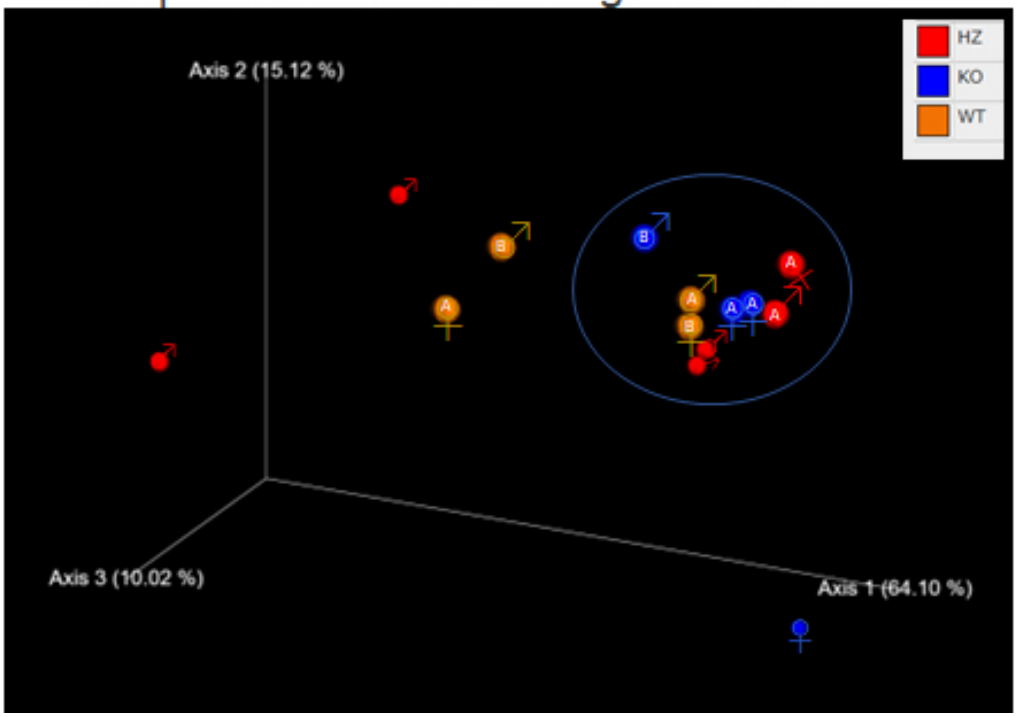


Figure 3.5 Breeding strategy to derive $C3^{KO}$ mice. $C3^{WT}$ were crossed with $C3^{KO}$ mice (P1) to produce $C3^{HZ}$ offspring (F1). From 2 $C3^{HZ}$ F1 breeding pairs, 2 F2 litters (A and B) totaling 14 mice were derived. The F2 offspring included 4 $C3^{HZ}$ (3 females, 1 male), 6 $C3^{WT}$ (3 females, 3 males), and 4 $C3^{KO}$ (2 females, 2 males). Separating mice from each litter by sex only resulted in $C3^{KO}$ mice sharing the cage with their $C3^{WT}$ and/or $C3^{HZ}$ littermates; however, due to the low number of $C3^{KO}$, there were $C3^{WT}$ and $C3^{HZ}$ mice that did not share a cage with $C3^{KO}$.

Considering there are no publications comparing this genotype's gut bacteria population with $C3^{WT}$ mice when co-housed, I thought to identify the bacteria recovered from stool samples of mice in our crossbreeding colony. One possibility is that the $C3^{KO}$ mice housed independently may be susceptible to IM because of unique bacteria that colonize the complement deficient gut. I obtained the 16S Ribosomal V3-V4 gene sequences from 14 stool samples including from all 3 genotypes. Stool is used as a surrogate measure for mucosal gut bacteria, understanding that there are differences. For a general comparison between the samples, I prepared an unweighted UniFracPCoA plot of the sequence data. Figure 3.6A shows that most of the mice cluster with the obvious exceptions being the P1 $C3^{KO}$ female and 2 male F1 $C3^{HZ}$. This pattern suggests that the two parents were quite different (presumably, data not available for the P1 $C3^{WT}$ male breeder), likely due to being kept in separate colonies. The bacteria in their F1 offspring are already becoming more similar. By the F2 generation the bacteria recovered in stool are even more similar, with all but 2 samples clustering with 2 of the F1 $C3^{HZ}$. It should be noted that all the F1 mice were males as the sequencing failed for female samples, so 2 of the F1 for which I have data were not used to breed to the F2 generation. It would be simple to presume that the 2 male F1 samples that cluster with most of the F2 were the fathers of the F2 litters but that cannot be confirmed with the data available. In terms of the samples in the cluster, mice from both litters and all 3 genotypes are present as are mice of both sexes. This means mice in different cages had similar gut bacteria. I interpret this result to mean that as $C3^{KO}$ and $C3^{HZ}$ shared a cage, their gut bacterial populations became more similar even if it meant diverging from the bacteria that were present in the colony founders. This level of comparison does not rule-out the possibility there are subtle differences between genotypes.

A


Emperor Ordination: unweighted UniFrac
HZ
KO
WT
Axis 2 (15.12 %)
Axis 3 (10.02 %)
Axis 1 (64.10 %)

B

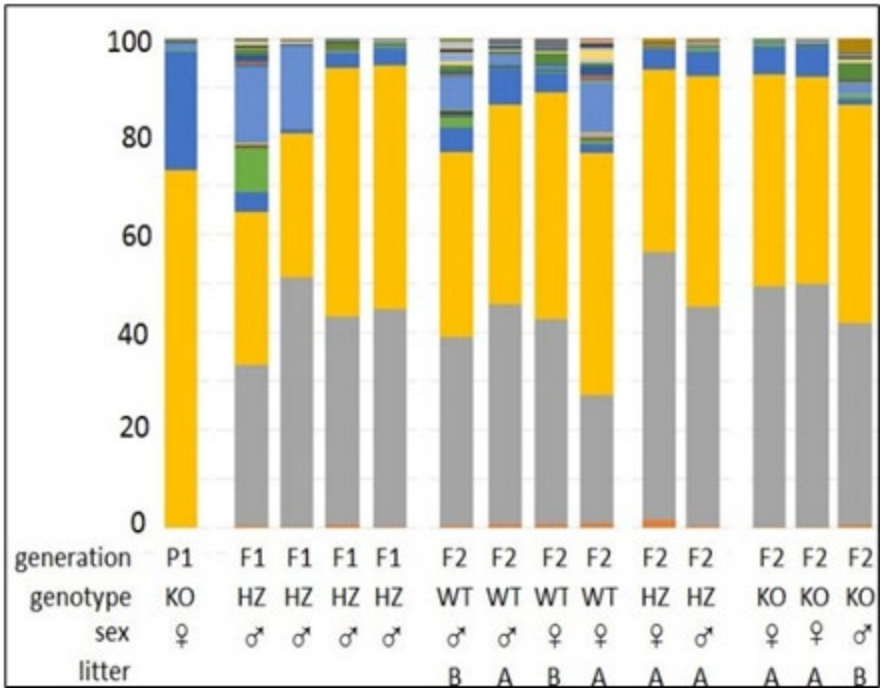

100
80
60
40
20
0
generation P1 F1 F1 F1 F1 F2 F2 F2 F2 F2 F2 F2 F2 F2
genotype KO HZ HZ HZ HZ WT WT WT WT HZ HZ KO KO KO
sex ♀ ♂ ♂ ♂ ♂ ♂ ♂ ♀ ♀ ♀ ♂ ♀ ♀ ♂
litter B A B A A A B

Figure 3.6 Gut bacteria from C3 deficient mice are similar to bacteria from wild type mice. **A** Unweighted UniFracPCoA plot of the sequence data, showing generation, sex, genotype and litter. Data was derived from 9 of the 14 mice in the F2 litters. **B** The relative microbial abundances within individual samples. The proportions are shown at the genus level. **C** The legend for B.

The relative abundances are shown at the genus level in Figure 3.6B. At this level of resolution, the $C3^{KO}$ female P1 sample is arguably the most different, followed by some samples seeming to have greater diversity representing roughly 10% of the bacteria. Figure 3.6B does not provide information on the absolute abundances, which may differ as well.

3.2.3. 5-fluorouracil-induced clinical illness symptoms and pathological phenotypes in C3 genotypes

Due to the relatively small number of F2 mice and blind to their genotype, all the offspring recieved 75mg/kg 5-FU in the 5 day treatment regimen. All genotypes lost weight during treatment period with the average maximum body weight loss at the end of the treatments reaching as low as 15%. In Figure 3.7 $C3^{KO}$ and $C3^{WT}$ mice lost weight at a similar rate (slope of the line) and to a similar extent during the treatment. Conversely, the $C3^{HZ}$ female mice did not lose weight over the first 2 days but lost weight on the final 3 days of the experiment, “catching up” with the other genotypes. Treatment with 5-FU resulted in soft stool pellets in all strains.

3.2.4. Lack of C3 does not protect jejunum from CIM

Untreated $C3^{KO}$ mouse jejunum was examined for any sign of inflammation. Figure 3.8 shows $C3^{KO}$ mice have uninflamed intestines. To evaluate whether C3, and consequently complement, have a role in our model of mucositis, I compared the H&E-stained jejunum histology of $C3^{KO}$, $C3^{HZ}$, and $C3^{WT}$ 5 days post 5-FU injections. Indeed, the jejunums of all mice exhibited inflammation. As shown in Figure 3.8A, the 3 genotypes exhibited comparable histopathological results consistent with IM, including reduced villus length, and the presence of focal ulcers. At the cell level, extensive epithelial damage and disarrangement of epithelial cells were observable, also, altered nuclear size and shape suggesting, pyknosis, karyorrhexis, and karyolysis, and enlargement of nuclei in much of

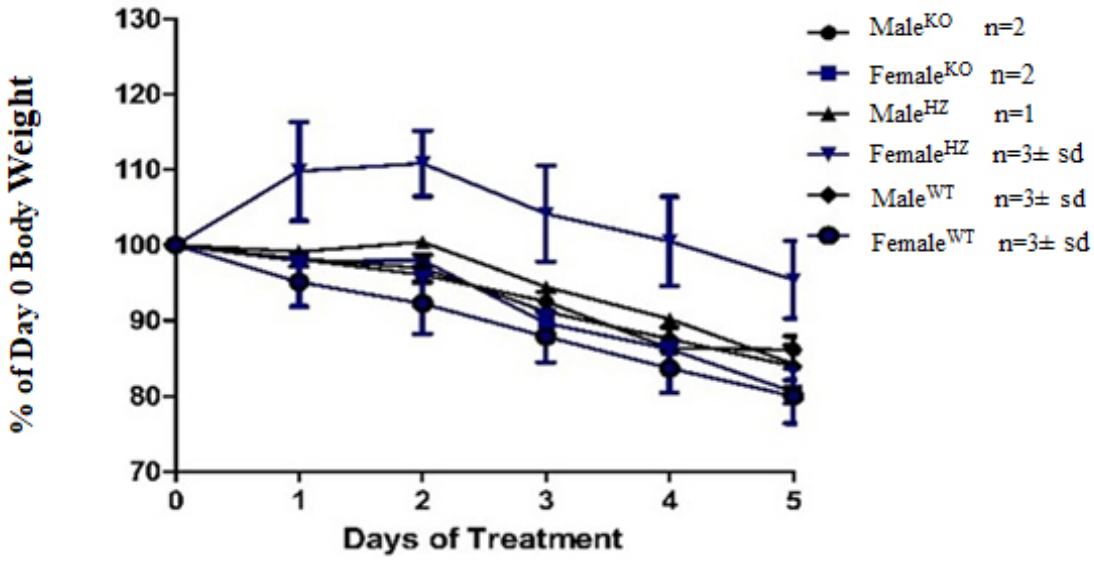


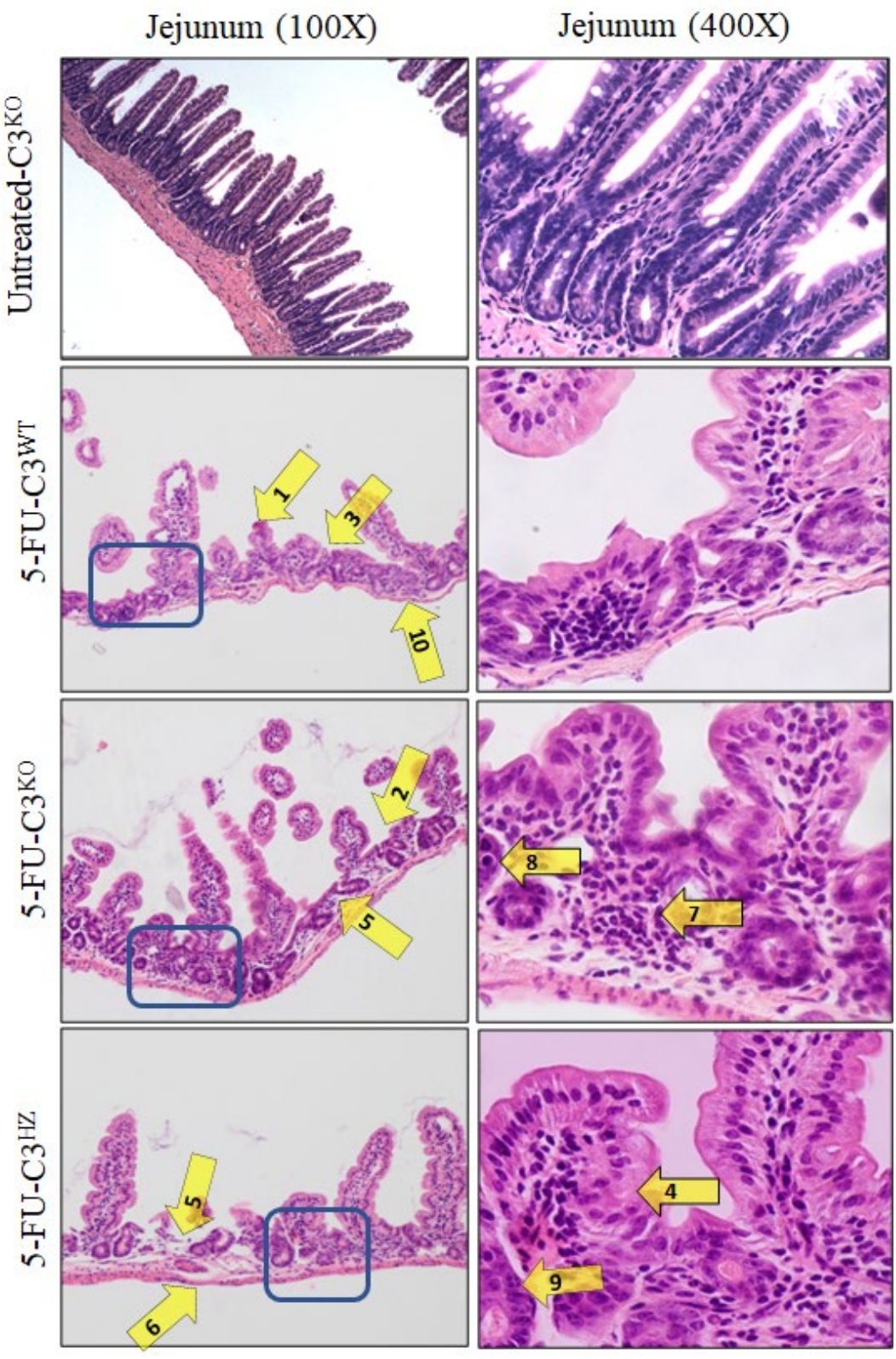
Figure 3.7 5-fluorouracil-induced clinical illness symptoms and pathological phenotypes in C3 genotypes. $C3^{KO}$, $C3^{WT}$, and $C3^{HZ}$ mice received a single dose of 75 mg/kg 5-FU (5 intraperitoneal injections), then 24 hours after the final injection mice were euthanized. Body weight was recorded daily. The end point of losing weight is 15%. (n= 4/each group). $C3^{KO}$ and $C3^{WT}$ mice lost weight at a similar rate. Female $C3^{HZ}$ mice lost weight on day 3, 4 and 5 of the experiment. sd= standard deviation.

the epithelium. Mitoses were observed in some epithelial cells. Crypts exhibited obvious architectural distortion in size and shape including crypt loss, crypt atrophy and large spaces between crypts. Other changes are dissociation between mucosal layers and between epithelial cells, moderate leukocyte infiltration in both the lamina propria and the crypts, and epithelial cell apoptosis hallmarked by clumped chromatin. Additionally, changes occurred in the smooth muscle layer, including atrophy. The jejunal pathological phenotypes were similar in the 3 genotypes (Figure 3.8B). Based on these histopathological features in the jejunum, $C3^{KO}$ mice experience CIM.

3.2.5. Lack of C3 does not protect the colon from CIM

Expanding on these results, as with the MBL^{KO} mice,Ievaluated whether the genetic deficiency of C3 protects the mouse colon from CIM. Untreated $C3^{KO}$ mouse colon was examined for any sign of inflammation. Figure 3.9A shows $C3^{KO}$ mice have uninflamed intestines.Icompared the H&E-stained colon samples of the three C3 genotypes treated with 5-FU for signs of inflammation. Shown in Figure 3.9A, the microscopic examination revealed all 3 genotypes contain similar pathological features, including extensive epithelial damage characterized by degenerative enterocytes, pleomorphic nuclear size and shape and orientation involving, pyknosis, karyorrhexis, and karyolysis, the presence of marked enlargement of nucleus in much of the epithelium, and no difference in the numbers of goblet cells (Figure 3.8B). Focal ulceration, mitosis, and apoptotic cells marked by condensation of chromatin were observed. Other observed signs of inflammation are damaged crypts, fusion at the top of crypts, cryptitis, a moderate inflammatory leukocyte infiltrate, edema, and vascular dilatation in the submucosa. These pathological features are compatible with mice losing weight and experiencing changes in stool consistency. contrasting jejunum samples, the colons were less inflamed by injections of 5-FU in mice.

A


Jejunum (100X)
Jejunum (400X)
Untreated-C3KO
5-FU-C3WT
5-FU-C3KO
5-FU-C3HZ
1
3
10
2
5
8
7
5
6
4
9

B

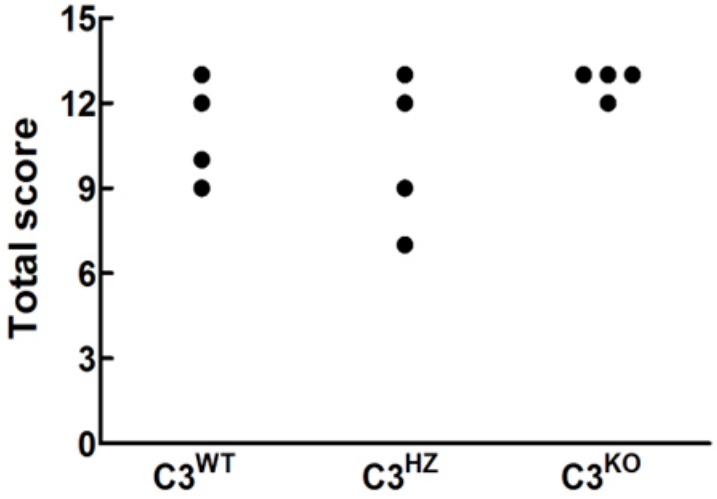


Figure 3.8 Neither the lack of C3 nor C3 heterozygosity protect the jejunum from mucositis. **A** H&E-stained photomicrographs of the jejunum prepared from untreated $C3^{KO}$ and 5-FU-treated $C3^{KO}$, $C3^{WT}$ and $C3^{HZ}$ genotype. Jejunum samples demonstrated severe villus height shortening (arrow 1), focal ulceration (2), extensive epithelial damage (3), altered nuclear size and shape and nuclear enlargement (4), obvious crypt architectural distortion in size and shape including crypt loss, crypt atrophy, and large spaces between crypts (5), dissociation between mucosa layers and between epithelial cells (6), moderate leukocyte infiltration in both the lamina propria and the crypts (7), apoptosis (8), mitosis (9), atrophy in the smooth muscle layer (10). Sections were examined using a LEICA DM1000 light microscope equipped with a LEICA MC170 HD camera. The blue box on left panels illustrate the region shown in the right panels at higher magnification. **B** Total pathology scores. Each dot represents a single mouse.

The conclusion is that $C3^{KO}$ and $C3^{HZ}$ mice experience CIM in their colons.

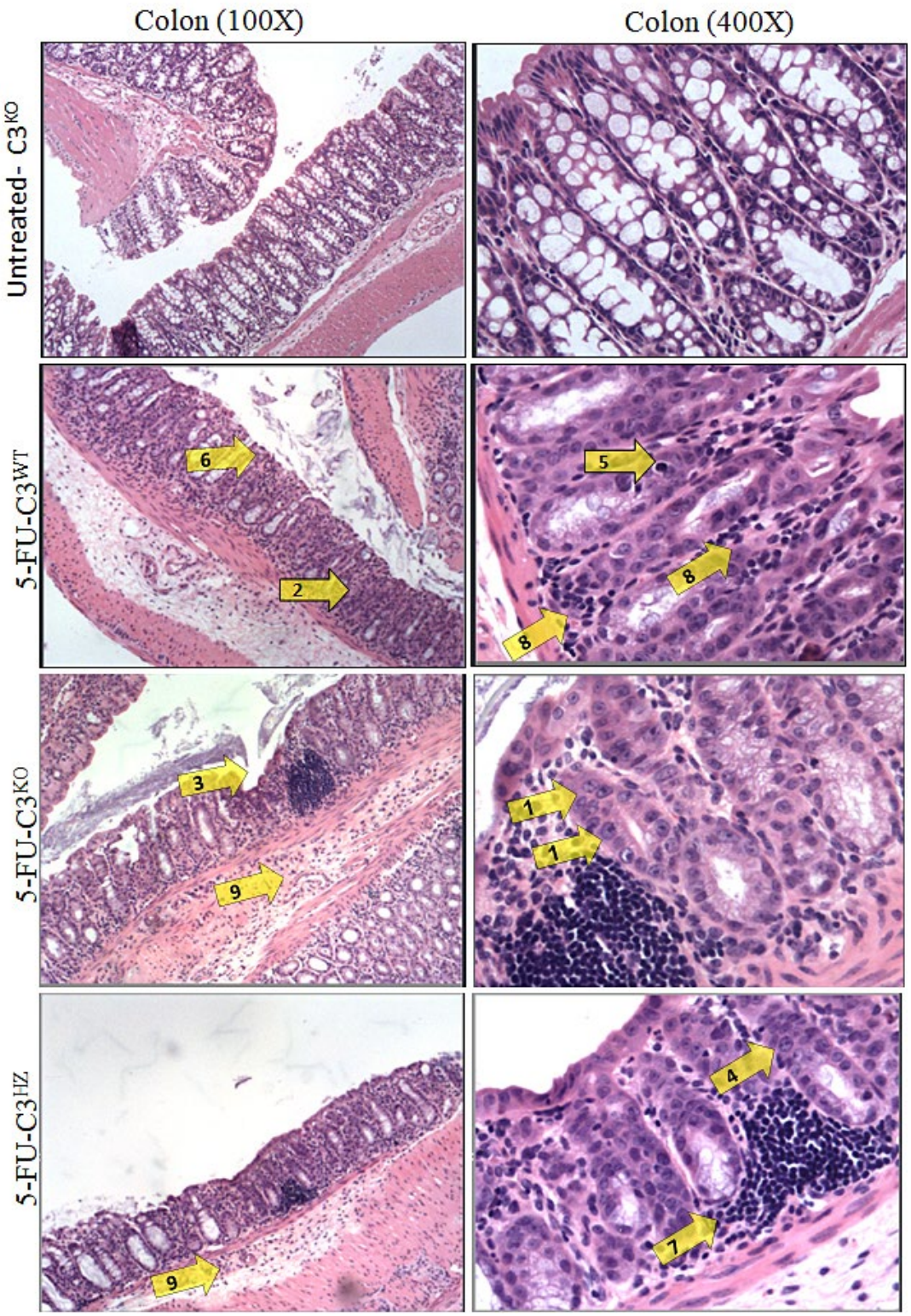
3.2.6. Neither C3 deficient nor heterozygous mice develop oral mucositis

$C3^{KO}$ mice developed IM so it was reasonable to examine the mice for OM. As seen with MBL^{KO} mice, the oral mucosa of $C3^{KO}$ mice is normal (Figure 3.10). I compared the buccal mucosa and ventral tongue samples of 5-FU- treated mice of the 3 genotypes. As shown in Figure 3.10, the buccal mucosa and ventral tongue show no evidence of inflammation in any genotype; the oral mucosa is intact with no ulceration, no epithelial hyperplasia, no increase in mitoses, and a lack of inflammatory infiltrate in the lamina propria. Despite the period of treatment, our results are suggestive that mice may not be overly susceptible to chemotherapy induced OM, at least when treated using our regimen.

3.3. Complement deficiency and methotrexate induced mucositis

MTX has a similar mechanism of action as 5-FU and there is value in confirming that OM and possibly IM are elicited by more than one drug with a history of causing CIM in humans. MBL^{KO}, MBL^{WT}, $C3^{KO}$ and $C3^{WT}$ mice were treated with a single daily dose of 75mg/kg-MTX for 5 days. All genotypes lost weight to the same extent, shown in Figure 3.11A. Unlike our observations using 5-FU, the consistency of day 5 MTX-treated mouse stool of both genotypes was dark black and dry pellets with some threads of white fibers on the outer surface. Nevertheless, administration of MTX for 5 days resulted in inflamed jejunums and colons. As shown in Figure 3.11B (MBL) and 3.11C (C3), the microscopic examination of the jejunum and colon revealed similar histopathological features as observed with 5-FU administration. Histopathological features were observed in the epithelium, and crypts. Despite clear evidence of IM, MTX administration did not elicited OM in any genotype of mouse.

A


Colon (100X)
Colon (400X)
Untreated- C3KO
5-FU-C3WT
5-FU-C3KO
5-FU-C3HZ
6
2
5
8
8
3
9
1
1
9
4
7

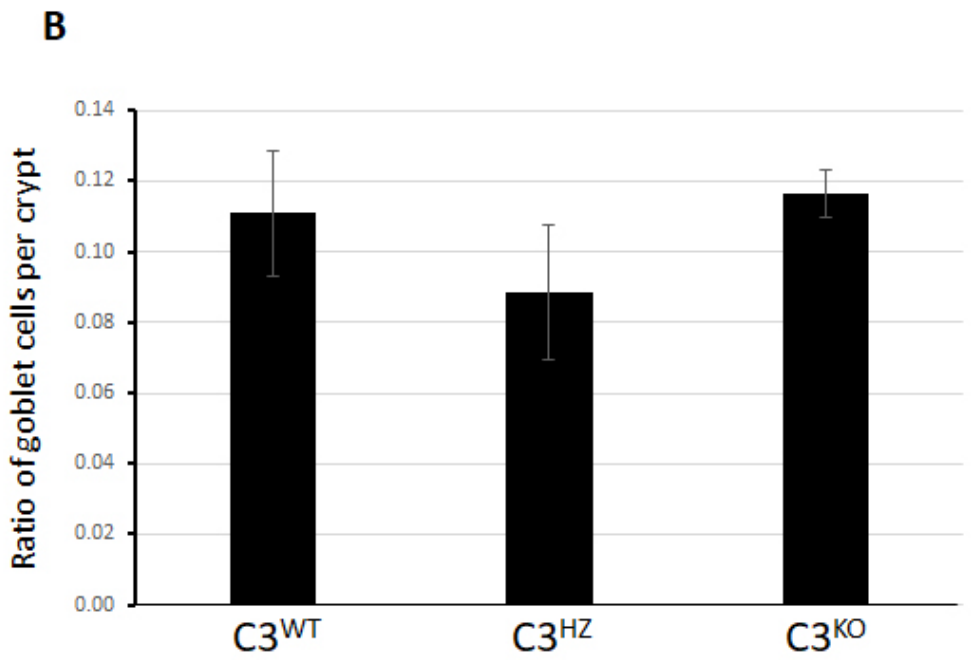


Figure 3.9 Neither the lack of C3 nor C3 heterozygosity protect the colon from mucositis. A Representative photomicrographs of the colon prepared from untreated and 75mg/kg 5-FU-treated $C3^{WT}$, $C3^{KO}$, and $C3^{HZ}$ mice. Colon samples demonstrated epithelial damage with pleomorphic nuclear size, shape, and orientation involving, pyknosis, karyorrhexis, and karyolysis, the presence of marked enlargement (arrow 1) reduced the numbers of goblet cells (2), focal ulceration(3), mitosis(4), apoptosis (5), damaged crypts and fusion at the top of crypts (6), cryptitis (7), moderate inflammatory leukocytes infiltrate the lamina propria and the crypts (8), edema, and vascular dilatation in submucosa(9). Sections were examined using a LEICA DM1000 light microscope equipped with a LEICA MC170 HD camera. **B** Numbers of goblet cells were similar in all genotypes treated with 5-FU. n= 4 ± standard deviation. (Compare with Figure 3.3 which shows untreated WT mouse goblet cell numbers are roughly 0.7 or 70% of cells in each crypt)

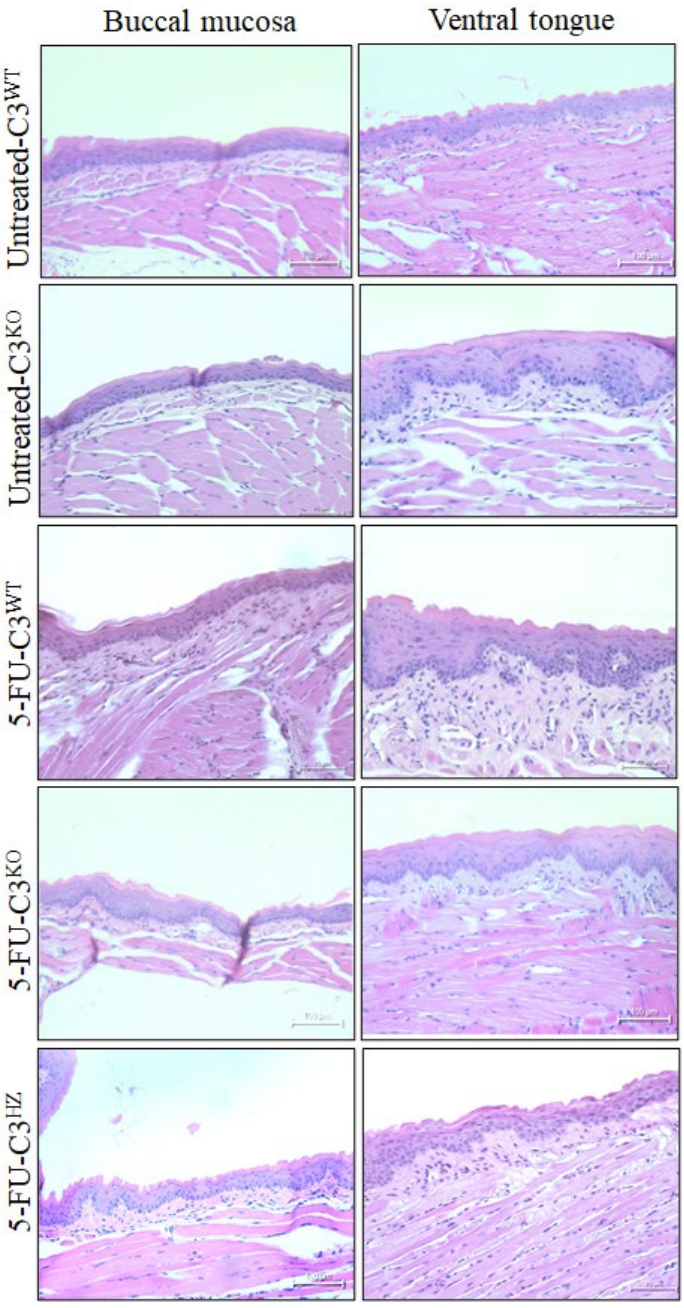
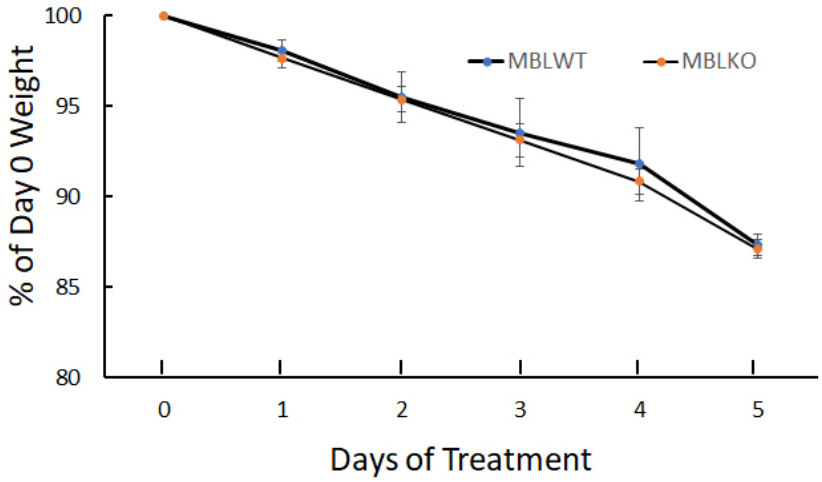
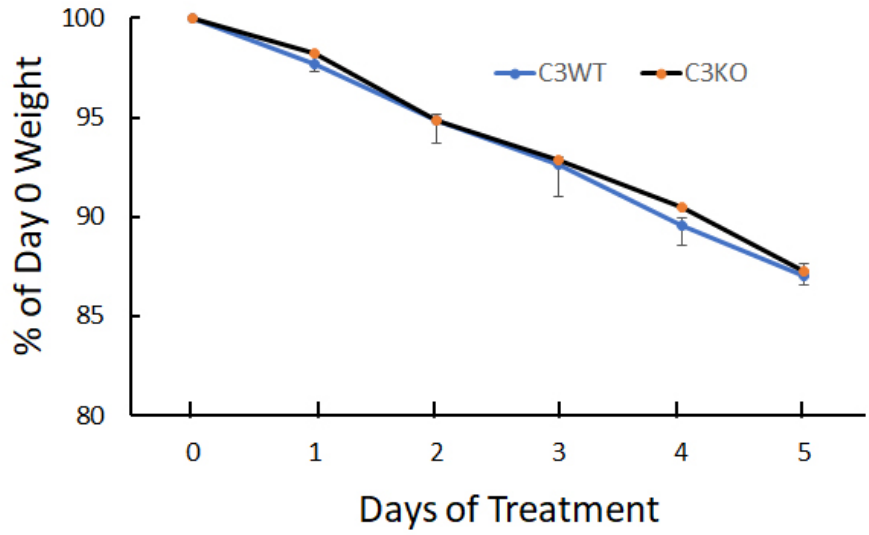


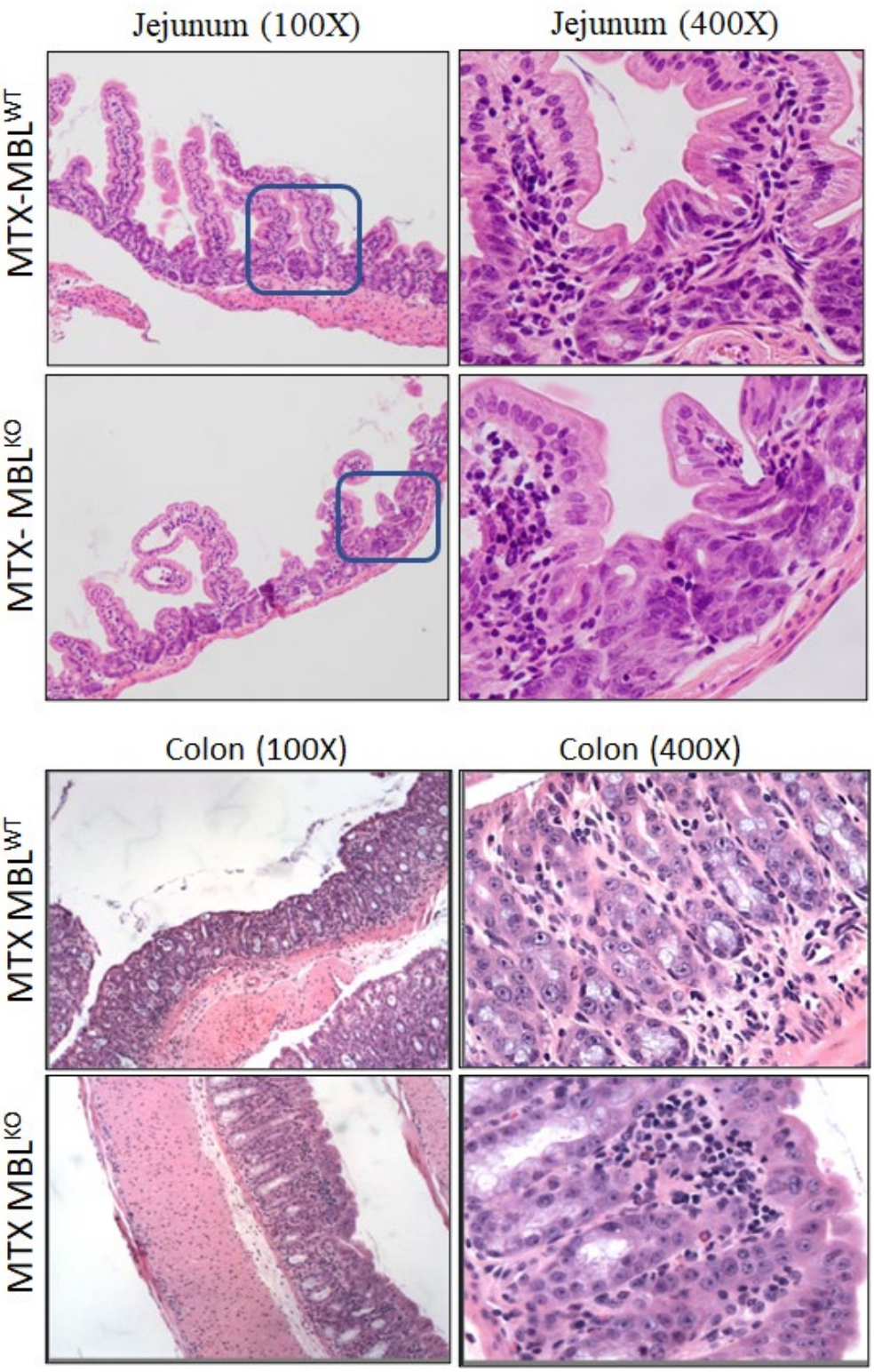
Figure 3.10 Neither C3 deficient nor C3 heterozygous mice develop oral mucositis. Representative photomicrographs stained with H&E comparing the buccal mucosa (left panels) and ventral tongue (right panels) of untreated $C3^{WT}$, $C3^{KO}$, and $C3^{WT}$, $C3^{KO}$ and $C3^{HZ}$ mice treated with 5 injections of 75 mg/kg of 5- FU (day 0 - 4) (n= 4/each group). There is no noted evidence of inflammatory changes in both tissues. Sections were stained with H&E and examined using a LEICA DM1000 light microscope equipped with a LEICA MC170 HD camera. 200X magnification.

A

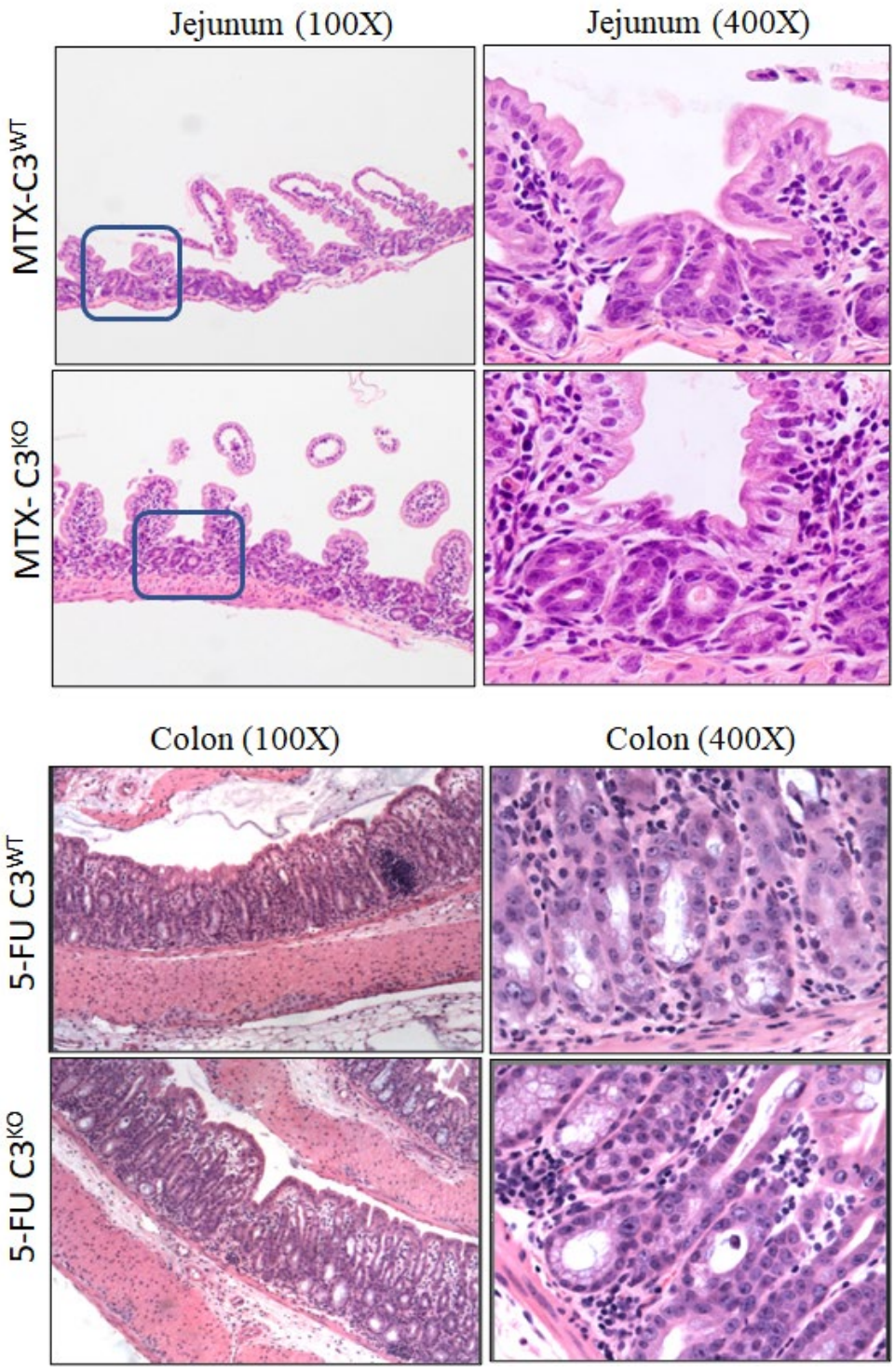

100
95
90
85
80
% of Day 0 Weight
MBLWT
MBLKO
0
1
2
3
4
5
Days of Treatment


100
95
90
85
80
% of Day 0 Weight
C3WT
C3KO
0
1
2
3
4
5
Days of Treatment

B


Jejunum (100X)
Jejunum (400X)
MTX-MBL^WT
MTX- MBL^KO
Colon (100X)
Colon (400X)
MTX MBL^WT
MTX MBL^KO

C


Jejunum (100X)
Jejunum (400X)
MTX-C3WT
MTX- C3KO
Colon (100X)
Colon (400X)
5-FU C3WT
5-FU C3KO

D

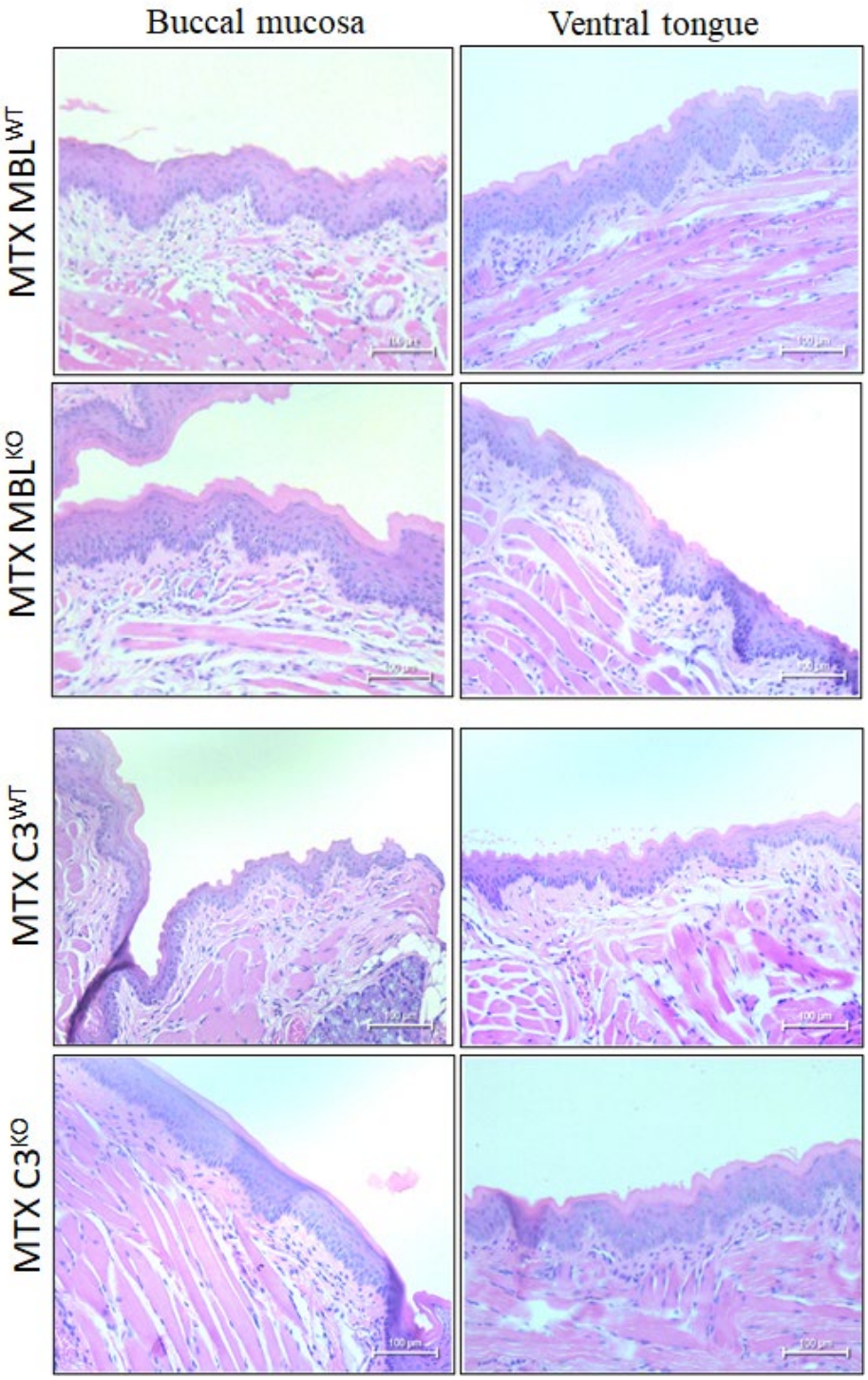
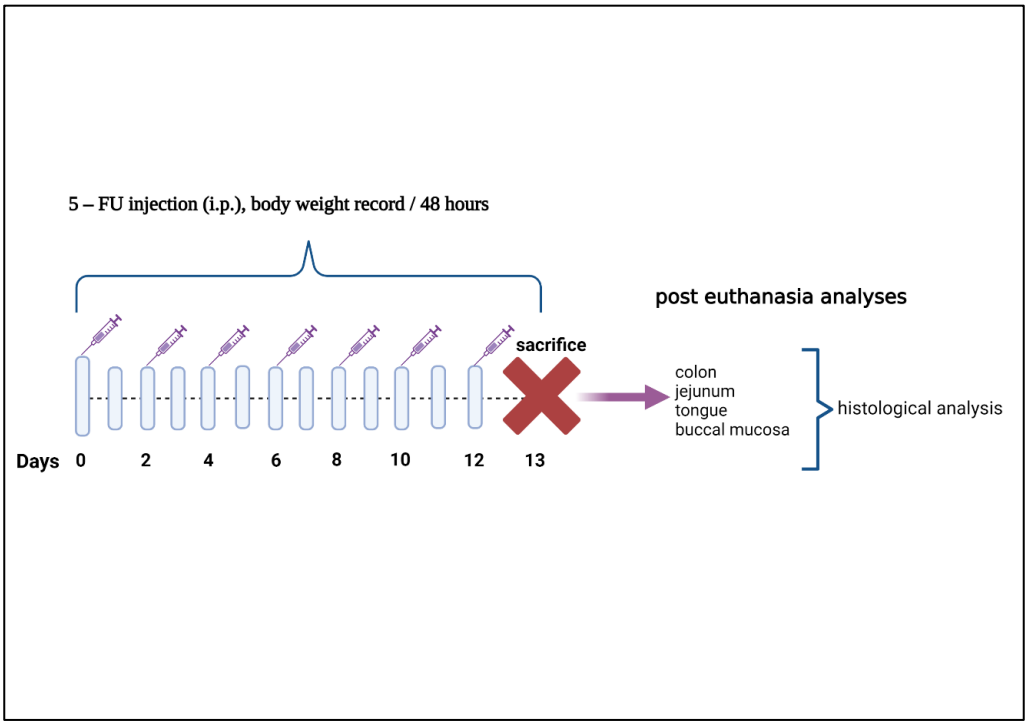

Buccal mucosa
Ventral tongue
MTX MBL^WT
MTX MBL^KO
MTX C3^WT
MTX C3^KO

Figure 3.11. Both MBL and C3 deficient mice develop IM but not OM following methotrexate injections. **A** Relative body weight of MBL^{WT}, MBL^{KO}, $C3^{WT}$ and $C3^{KO}$ mice treated with 5 injections of 75 mg/kg of methotrexate (days 0 - 4) (n= 4 ± standard deviation/genotype). **B** Representative photomicrographs stained with H&E showing jejunum and colon of MBL^{WT} and MBL^{KO}. **C** jejunum and colon samples of $C3^{WT}$, $C3^{KO}$ mice. Features of the pathology are similar to those described as due to 5-FU. **D** H&E photomicrographs showing the buccal mucosa (left panels) and ventral tongue (right panels) of MBL^{WT}, MBL^{KO}, $C3^{WT}$ and $C3^{KO}$ mice. No evidence of inflammatory changes in the buccal mucosa or ventral tongue sections in all treated genotypes. Sections were examined using a LEICA DM1000 light microscope equipped with a LEICA MC170 HD camera. 200X magnification.

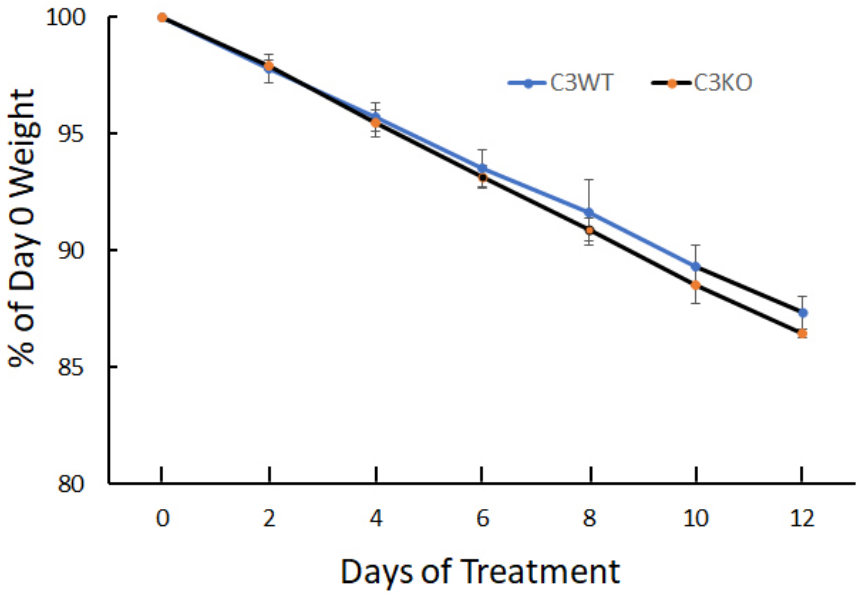
3.4. Extending the time mice are treated with 5-FU does not lead to oral mucositis

The regimen of a single daily dose of 75mg/kg drug for 5 days (5 IP injections) has not elicited OM in any strain of mouse. There are at least 2 considerations when thinking about adaptations to the regimen that may lead to OM. First, any higher dose would result in the mice losing over 15% of their starting weight, triggering an early endpoint. Secondly, the epithelium of the oral mucosa is replaced/replenished much slower than the intestinal epithelium and more time accumulating apoptotic cells may be necessary to trigger inflammation. Thus, I sought to extend the period of treatment to 2 weeks using a dose of 75mg/kg 5-FU every 48 hours (total of 7 IP injections). The regimen ends on day 13 when the mice are euthanized (Figure 3.12A). The mice lost weight (Figure 3.12B). As shown in Figure 3.4.C and D, the microscopic examination of the jejunum and colon shows similar histopathological features as observed with a 5-FU administration regimen for 5 days. Histopathological features were observed in the epithelium and crypts. When the oral mucosa of mice treated using this regimen was examined it was apparent the mice had not developed OM (Figures 3.12E). I concluded that in our model of mucositis, prolonging the time mice treated with 5-FU intraperitoneally does not lead to oral mucositis.

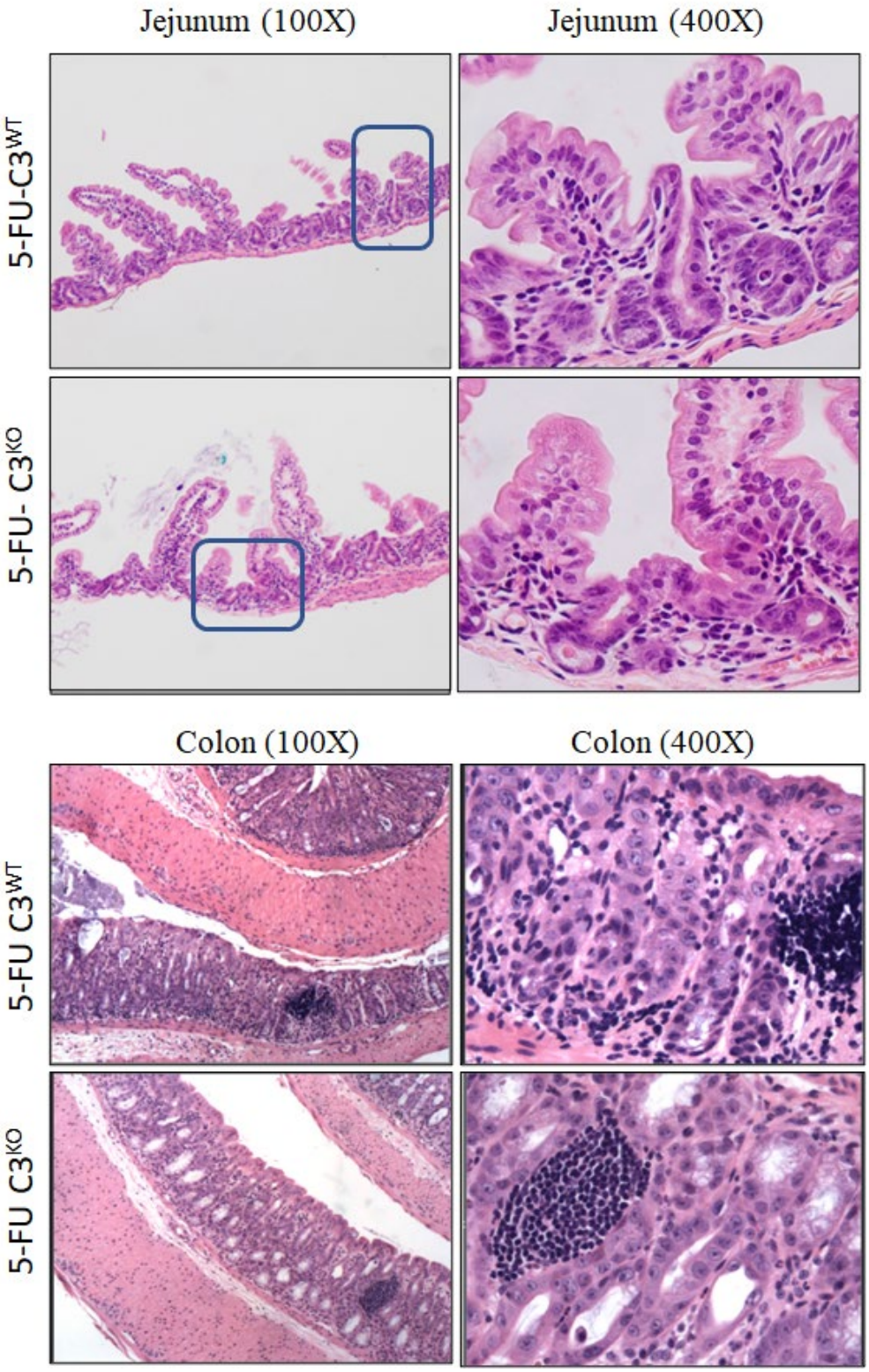
A


5 – FU injection (i.p.), body weight record / 48 hours
post euthanasia analyses
sacrifice
colon
jejunum
tongue
buccal mucosa
histological analysis
Days 0 2 4 6 8 10 12 13

B


C3WT
C3KO
% of Day 0 Weight
100
95
90
85
80
0 2 4 6 8 10 12
Days of Treatment

C


Jejunum (100X)
Jejunum (400X)
5-FU-C3WT
5-FU- C3KO
Colon (100X)
Colon (400X)
5-FU C3WT
5-FU C3KO

D

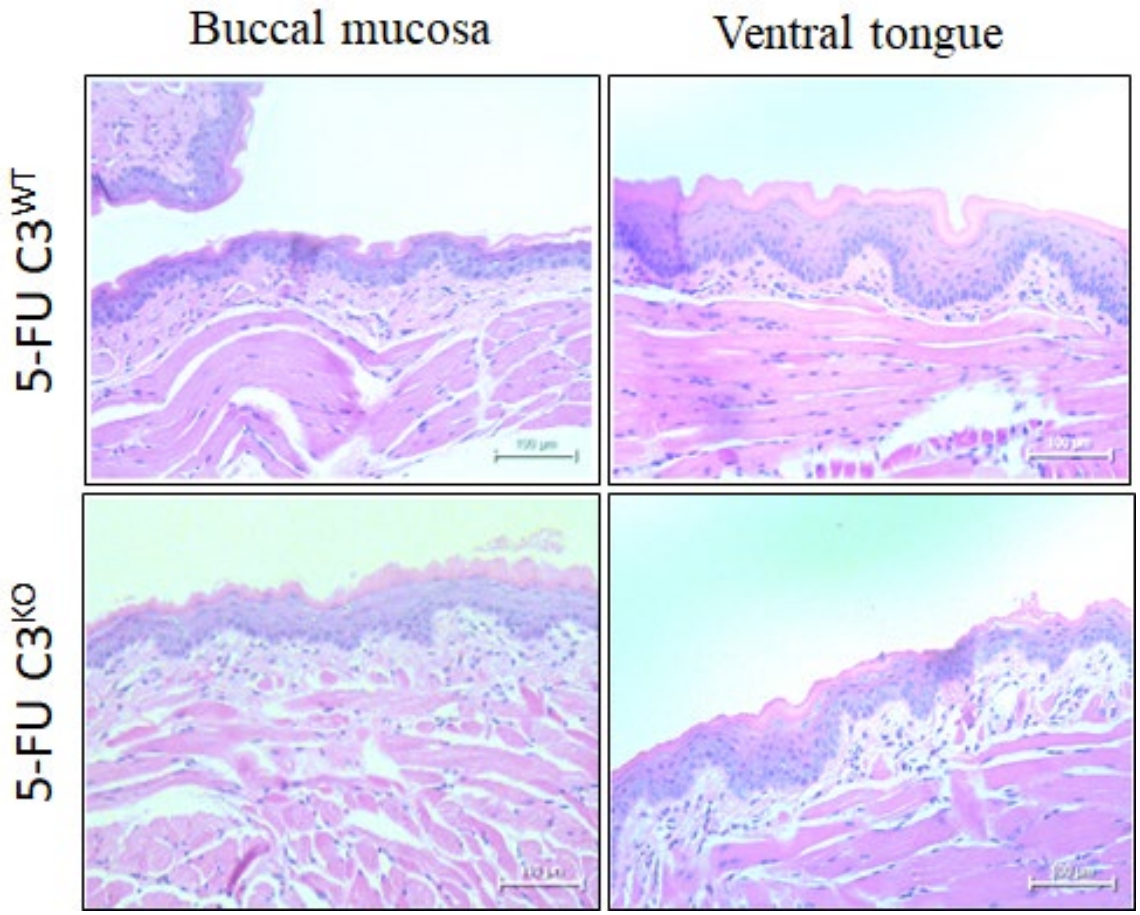


Figure 3.12 Extending the time mice are treated with 5-fluorouracil does not lead to oral mucositis. **A** Schematic study design. $C3^{WT}$ and $C3^{KO}$ mice were treated for 2 weeks (days 0 - 12) with intraperitoneal injections every 48 hours (75 mg/kg 5-FU). The mice were euthanized on day 13 (n= 4/each gentotype). **B** relative body weight. **C** representative photomicrographs stained with H&E showing jejunum and **D** colon samples. Features of the pathology are similar to those described as due to the 5-FU administration regimen for 5 days. **E)** H&E photomicrographs comparing the buccal mucosa (left panels) and ventral tongue (right panels) of $C3^{WT}$ and $C3^{KO}$mice. No inflammation was observed in the buccal mucosa or ventral tongue sections. Sections were examined using a LEICA DM1000 light microscope equipped with a LEICA MC170 HD camera. All panels magnification is 200X.

CHAPTER 4. DISCUSSION

4.1. Complement and IM

4.1.1. Complement in the digestive tract

Despite gains in survival following cancer treatments, adverse effects continue to inflict considerable morbidity on a high number of patients (Al-Dasooqi et al., 2013; Chang et al., 2012). OM, in particular, can be highly debilitating yet is likely underreported (Pulito et al., 2020). While there have been efforts to prevent or treat OM in patients, less research has been invested in mitigating IM in patients and most studies have been conducted in rodents (Huang et al., 2022). Noteworthy is the fact that most experimental findings made in rodents and involving specific mediators have not been applied to OM, making it less clear whether the inflammation along the digestive tract is necessarily similar. Nor have the efforts in the clinic to mitigate OM been applied to IM. Nevertheless, the pathogenesis of mucositis generally is becoming more clear (Keefe 2004; Keefe et al., 2007).

Mucositis is understood to be the product of mucosal and submucosal signaling disturbances arising from a large number of apoptotic cells (Gibson et al., 2003; Paris et al., 2001). Whether complement, appreciated to be inflammatory, is engaged in any or all stages of these processes is not known. The activation of complement contributes to inflammation in multiple ways. Split complement molecules opsonize microbes but also unwanted cells, the anaphylatoxins are inflammatory through activities on leukocytes and other cells (Cao et al., 2012) and the MAC leads to cell lysis, possibly resulting in the release of DAMPs (Lambris et al., 2010).

Why presume complement is active in the digestive system? Complement proteins are made by most epithelial cells (Sünderhauf et al., 2017) and split effector molecules are found on the mucosa in multiple digestive diseases and even in the intestinal lumen (Andoh

et al., 1993; Riordan et al., 1997). Many immune cells have recently been discovered to synthesize C3 and C5, and "self-activate" C3 and C5 intracellularly by the cytoplasmic proteases cathepsin L and B, leading to C3a and C5a secretion (discussed in greater detail later) (Kopf et al., 2002; Liszewski, et al., 2013; Minton, 2014; Zhang et al., 2018a). In addition, pancreatic acinar cells secrete C3 into pancreatic secretions which allows complement molecules to enter the lumen of the small intestine (Sina et al., 2018). Indirect evidence for activation of complement in the lumen is the finding that colonic and other epithelial cell lines express anaphylatoxin receptors on the apical surface (Cao et al., 2012; Fayyazi et al., 2000). More directly, there are multiple reports showing split components on the mucosa. C3a, C3b, and C3d, have been found on the colonic mucosa of healthy mice and levels of these proteins were considerably increased in a chemical model of colitis. Patients with Crohn's disease or ulcerative colitis were shown to have increased C3b and MAC deposition on the apical surface of the small and large intestinal epithelium (Halstensen et al., 1989; Halstensen et al., 1992; Ueki et al., 1996). Perhaps the most compelling evidence for activated complement in the lumen is the report that bacteria recovered from mouse stool samples are coated with split C3 molecules (Kirkland et al., 2012). So, while there is evidence for complement activation in the intestines, what might be the stimulus/activator in mucositis?

4.1.2. Complement and apoptosis

Apoptosis is a crucial, normal, mechanism in development and tissue homeostasis and it also takes place in areas of ongoing inflammation (Kang et al., 2012; Ravichandran 2003; Savill & Fadok 2000). Apoptosis is an early damage indicator and a hallmark histological manifest of intestinal damage and mucositis (Keefe 1997; Keefe et al., 2000). Apoptosis and proliferation play a role in the integrity of the intestinal epithelium after

chemotherapy (Keefe 1998). Thus, apoptotic cells must be rapidly cleared by phagocytes to avoid tissue injury and inflammation brought on by the release of immunogenic intracellular molecules (Savill et al., 1993; Savill & Fadok 2000). Complement activation by dying cells and types of proteins associated with dying cells is known to occur (Trouw et al., 2008). Multiple studies have documented the clearance of cellular debris and apoptotic cells by complement through phagocytes (Mevorach et al., 1998; Kang et al., 2012) and complement deficiency was found to hinder or prevent this process (Taylor et al., 2000). The alternative and classical pathways both play a role in apoptotic cell clearance by phagocytes (Matsui et al., 1994; Mevorach et al., 1998; Tsuji et al., 1994). In steady state conditions, the majority of apoptotic cells will be eliminated before the late stage; however, when there are excessive numbers of apoptotic cells as in mucositis, a reduction of phagocytic capacity, or poor phagocytosis, the apoptotic cells may persist in tissues (Trouw et al., 2008). During this stage apoptotic cells acquire complement components that enhance recognition and removal. Properdin and C1q interact directly with dying and dead cells (Trouw et al., 2008). The study by Jain et al., (2017) dismissed properdin in the activation of complement since C3a and C5a levels were similar between properdin deficient and WT strains of mice treated with 5-FU; however properdin deficient mice were significantly protected from IM indicating properdin is likely also flagging apoptotic cells. MBL and other lectins may also attach to late apoptotic and necrotic cells (Nauta et al., 2003; Nauta et al., 2004; Vandivier et al., 2002), providing a compelling reason to predict the lack of MBL will affect CIM, whether through complement activation or not. This understanding implicates MBL as a possible link between apoptosis and inflammation during mucositis. While I did not provide evidence for MBL binding to apoptotic cells, MBL^{KO} mice were as susceptible as MBL^{WT} suggesting neither MBL1 nor 2 link apoptosis

to mucositis.

4.1.3. Complement and mucositis

Despite rich evidence that complement is active in the digestive tract and that apoptotic cells can launch complement, reports examining complement during mucositis are scarce. Bowen et al., (2007) reported that rats given a single injection of irinotecan, a chemotherapeutic that blocks topoisomerase 1, had increased mRNA expression of factor D, C1q and C2, indicative of an acute phase response, while C3 expression, also an acute phase reactant, was not elevated. The source of the complement molecules was undetermined and it was unknown if complement was involved mechanistically in the mucositis. Jain et al., (2017) showed complement becomes activated during IM, as both C3a and C5a were elevated in homogenates prepared from jejunums of 5-FU-treated mice; however, they dismissed any role for C5a as a C5aR1 antagonist failed to limit the inflammation or protect mice from IM. Again, in the Jain et al. (2017) study, properdin, presumed to stabilize the alternate pathway C3 convertase, was pro-inflammatory since deficient mice were significantly protected despite the elevated anaphylatoxin levels. Therefore, mucositis is properdin-dependent but the protection in this strain is independent of complement activation. Lacking properdin, the presumption was that the alternative pathway to complement activation was quenched, ruling-out the alternative pathway in mucositis.

4.2 Complement deficiency and intestinal mucositis

4.2.1 Mannose Binding Lectin, intestinal complement and mucositis

If indeed the alternative pathway is disabled in properdin deficient mice yet complement becomes activated, then another route to activation must be active. I chose to next examine the lectin pathway, as it is unlikely that the classical pathway, with a

requirement for immune complexes, is activated. The lectin pathway and consequent inflammatory processes are activated by MBL (Choteau et al., 2016; Takahashi 2011; Thiel et al., 2006). The gastrointestinal tract expresses both MBL-A and MBL-C in mice and MBL2 in humans (Choteau et al., 2016). It has been demonstrated that MBL is produced locally by intestinal epithelial cells in response to local inflammation and it is even considered critical in gut homeostasis (Choteau et al., 2016; Held et al., 2008), in which case untreated MBL^{KO} mice might be expected to have some pathology. I did not observe any pathology in the intestines of untreated MBL^{KO} mice. This history of findings and the earlier evidence that MBL may bind late apoptotic cells, provides compelling evidence to predict that MBL would activate the lectin pathway during mucositis. However, MBL deficient mice proved to be as susceptible to mucositis as WT, providing the mice were co-housed prior to injecting with the chemotherapeutic drug. Thus, I ruled-out the MBL/lectin pathway to complement activation as contributing to mucositis; however, it is noteworthy that neither C3a nor C5a levels were measured so there remains a possibility that in fact complement was not activated. Furthermore, MBL is only one lectin among others that activate the lectin pathway. Ficolins and collectins are other lectin pathway activators. Further study needs to be done, for example, using MASP deficient mice before the lectin pathway can be entirely dismissed.

4.2.2 C3 deficiency and mucositis

With evidence that neither the alternative (Jain et al., 2017) nor the MBL pathways are critical to mucositis, I thought to remove the single-most important molecule in the network, C3. All three pathways to activation converge at C3 cleavage, in fact, AP activation requires C3, so the absence of C3 should absolutely quench AP activation. My experiments showed that $C3^{KO}$ mice experience IM similar to $C3^{WT}$. It has been reported

that the lack of C3 reduced alternative pathway mediated tissue damage (hemolysis in the study) but did not prevent classical pathway activation and the classical pathway C3 convertase (C4b2b) continued to be formed and cleaved C5 with low efficacy, leading to MAC and hemolysis (Zhang et al., 2019). Whether a similar, smoldering activation of C5 was occurring in $C3^{KO}$ mice could be addressed by measuring C5a, but it has already been established that C5a is not critical to mucositis (Jain et al., 2017). Additionally, it would be useful to stain for MAC, which could be contributing to cell death in the model (Jain et al., 2015).

4.3 Interactions between complement and gut bacteria

The intestinal epithelium acts as a sentinel for microbial activity in the lumen and as a physical barrier to limit luminal microbes from accessing the mucosa. In fact, the entire digestive system is colonized, potentially by all types of microbes, and different regions of the tract may host different microbes (Biswas & Rahaman 2020; Guarner & Malagelada 2003). The most comprehensively studied are bacteria, which can be beneficial or harmful, some are even opportunistic pathogens, meaning the bacteria become harmful only in dysbiotic states (Al-Dasooqi et al., 2013; Hamouda et al., 2017; Kim et al., 2020; Nakata et al., 2013). Indeed, there is evidence that gut microbes impact immune development including complement synthesis by host cells (Huang et al. 2023) and that multiple host immune factors, including complement, influence the bacteria that colonize the gut (Khan et al. 2019). Complement activation in the digestive tract, even during disease, may be due to microbes particularly bacteria, which point to AP or LP activation. Depletion of C3 levels reportedly exacerbates sepsis during acute gastrointestinal injury (Ye et al., 2019; Yuan et al., 2012a,b; Yuan et al., 2011); however, if $C3^{KO}$ mice became septic during my CIM regimen they may have been expected to lose weight faster than $C3^{WT}$ mice.

The relationship between host and gut microbes confounds studying diseases of the gut and there is a history of investigations using gene deficient animals and not controlling for their gut microbiomes by mixing the deficient mice with control mice. In such studies, a disease phenotype is described in the deficient strain that differs from WT until they are co-housed, then the phenotypes become similar (Khan et al., 2019). There are approaches to reducing the effect of mice raised with different gut microbiomes. A fecal transplant could be done between the strains. More straightforward, co-housing the mice results in the animals sharing their gut microbes through their feces and presumably "normalizing" both strains in the cage to become colonized by the same microbes (Singh et al., 2021). It is a very strong phenotype when a gene deficient mouse strain can sustain commensals very different from the WT strain while in the same cage. An alternative approach is to breed the two strains through several generations, the approach taken in this study for the C3 strains. Our analyses of the stool bacteria in progeny of KO x WT crosses demonstrated that the gut bacteria converged on a common profile with successive generations. Therefore, arguably, the gut bacteria were not a profound confounding factor in the mucositis when comparing F2 $C3^{WT}$ with $C3^{HZ}$ and $C3^{KO}$ mice. That the $C3^{KO}$ mice lost weight similar to $C3^{WT}$, is an indication that the deficient mice likely did not experience heightened bacterial dissemination from the intestines, otherwise the mice may have heightened illness. Finally, all the mice showed similar intestinal histopathology. Considering neither MBL^{KO} nor $C3^{KO}$ mice showed a mucositis phenotype different from WT mice indicates that these complement molecules do not contribute mechanistically to mucositis.

4.4 Complement may be anti-inflammatory

Up to this point, I was working on the presumption that activation of complement

would be pro-inflammatory. The fact that even $C3^{KO}$ mice showed no reduction in mucositis may point to the possibility that complement in this context is in fact anti-inflammatory or at least contributes to recovery and repair in the gut. This idea is supported by emerging research showing that many cells synthesize C3 and C5, cleave both using cathepsins, and release the anaphylatoxins which act back on the cells as growth factors. Other precedents support the idea that complement, although not activated through the traditional pathways, support cell growth. One example is intracellular C3 activation in Paneth cells promoting the normal growth and repair of the intestinal epithelium (Zhang et al., 2018b; Ricklin & Lambris 2013; Ye et al., 2019). Additionally, proliferation of stem cells and villi regeneration reportedly depends on C3a/C3aR1 signaling, demonstrated directly using mouse intestinal organoids (Matsumoto et al., 2017). Both $C3^{KO}$ mice and C3a receptor (C3aR) 1-deficient mice had dramatically compromised intestinal organoid formation (Matsumoto et al., 2017). C3 was found in *Lgr5*+ intestinal stem cells in organoids and intestinal crypts, indicating that intestinal stem cells can provide C3a to support autocrine proliferation (Matsumoto et al., 2017). Moreover, C3a through the C3aR1 protects injured tissues from oxidative damage by recruitment of mesenchymal stem cells (Schraufstatter et al., 2009; Matsumoto et al., 2017). It would be interesting to study the recovery from mucositis in the $C3^{KO}$ mice, which if autocrine C3a is important, should be compromised.

4.5 Oral mucositis and the drug dosing regimen

Under no condition did any strain of mouse used in this study experience OM. In fact, it seems to be common to experiments in which mice are given IP drug injections, that the mice fail to develop OM (Huang et al., 2022; Bertolini et al., 2017).

The proximity of the drug to the intestinal mucosa, the epithelium type (stratified

squamous epithelium versus simple columnar), and the different rates of epithelial turnover (1-4 days for small intestinal epithelium versus 14–21 days for buccal mucosa) may all contribute to increased susceptibility of the intestinal mucosa compared to the oral mucosa (Squier & Kremer, 2001). Thus, studying oral mucositis in mice is challenging since IM typically occurs before any oral lesions appear (Huang et al., 2022).

Since oral mucositis was not achieved using drug injections over 5 days and considering the slower turnover of oral epithelial cells, I sought to extend the period of treatment, to 2 weeks (IP injection every 48 hours). The frequency of drug injections had to be reduced or the mice risked reaching an early endpoint and not completing the regimen. Despite the longer exposure to 5-FU (MTX was not examined) microscopic examination demonstrated IM but no OM. My findings are indicative that mice and perhaps the C57BL/6 strain in particular, may not be susceptible to chemotherapy induced OM. Considering the convenience behind using mice and the reagents available compared to other rodents, it is worthwhile conducting a mouse strain comparison looking for OM susceptibility.

Contrasting the lack of OM following IP drug injections, and published while these experiments were being conducted, an IV administration regimen of 5-FU in C57BL/6 mice every 48 hours for 2 weeks reportedly resulted in IM and lesions in the oral cavity, included atrophy of the oral and esophageal epithelium, reductions in cell proliferation, increased apoptosis in the oral epithelium, and neutrophil infiltration consistent with OM (Chang et al., 2015; Bertolini et al., 2017). I did not adopt the IV regimen since the multiple IP injection regimen had been shown to cause IM with complement activation. It has not been proven that complement becomes activated following IV injections.

Another consideration, which has not been thoroughly examined, is whether there

are mouse strains more predisposed to develop OM, a line of research for future studies. In this vein, previous studies showed that IL-10 deficient mice (IL-10^{KO}) are highly sensitive to 5-FU -triggered IM (Jain et al., 2017). Another group had earlier reported that the strain was highly sensitive to IM triggered by MTX (de Koning et al., 2006). Neither study examined the animal's oral mucosa. To determine if IL-10^{KO} mice experience OM by 5-FU or MTX, including by IP injections, remains to be discovered.

4.6 Limitations

The regimen has limitations including the fact that the regimen does not resemble the treatment regimen used in humans. Chemotherapy in humans is applied in cycles, typically IV, and often combined with other drugs. The strategy is to build up the concentration of drug in the blood, have it impact the cancer, then the patient recovers. We used repeated drug injections which, considering the half-life of the drugs, concentrations likely declined between injections. Moreover, these repeated doses likely confuse the sequence of events related to the first 3 stages of mucositis as described by Sonis, by re-starting the cycle with successive injections. The regimen also used IP injection and we now know (or have evidence) that IV injections can lead to OM in mice without an additional physical injury to the oral mucosa. Interestingly, even the IV route required repeated IV injections to achieve OM.

The regimen did not include the mice harboring cancer. Ideally, a model of treatable cancer would be used and there are published precedents (for example, Guabiraba et, al., 2014). Of course, whether MBL or C3 deficiency affected the growth of the cancer would have to be determined as well. Furthermore, the combination of illness due to the cancer and drug could lead the mice to reach an early endpoint, even at drug doses too low to trigger mucositis. Finally, such a model is unlikely to trigger OM, based on our experience.

More specific observations of cell types and mediators, for example, levels of anaphylatoxins may have revealed differences between genotypes. If I had measured anaphylatoxins (C3a, C5a) and both happened to be elevated, I could have concluded that complement activation is MBL-independent. On the other hand, if both C3a and C5a are absent then complement activation is MBL-dependent and mucositis is complement - independent. Further conclusions could be made if C5a had been measured in the C3 experiment; if C5a levels were increased, complement activation is C3-independent whereas if C5a is absent then complement activation is C3-dependent and mucositis is complement-independent. This additional step means my conclusion over the contribution of complement activation is incomplete, and I can only conclude that MBL and C3 are expendable in IM.

The numbers of mice for which bacterial sequences were recovered was too small to raise the confidence in our conclusion that the gut bacteria became more similar with each generation, or to be confident in any sex-dependent differences.

Finally, the number of mice available for the C3 experiment was limited. There were too few mice to include untreated animals of any genotype or of either sex. This is important to ensure that the inflammation that was observed in HZ or KO mice was entirely due to the treatment and not due to bacteria the mice acquired while sharing the cage with WT. Presumably bacteria that cannot colonize the KO intestine yet is continuously ingested because the mice share the cage with HZ or WT animals could affect the intestine of a KO mouse.

4.6 Conclusion

Proceeding from the fact that mucositis development has complex pathobiology involving a variety of signaling pathways, my study aimed to investigate the possibility

that complement plays a role in model CIM. The key clinical and histopathological findings of the jejunum, colon, and oral mucosa of C3 and MBL deficient mice following 5-FU and MTX administration demonstrated that these complement molecules do not contribute mechanistically to the inflammation of CIM. Further investigation is required to more comprehensively understand the underlying pathobiological mechanism of mucositis. This will provide points along the inflammatory pathway and could contribute to treating this condition, for an effective therapy to be developed which can serve a high proportion of cancer patients.

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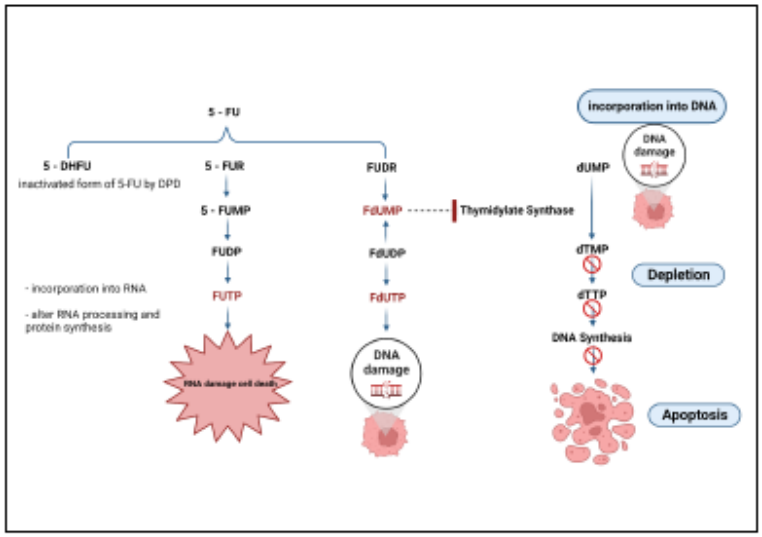

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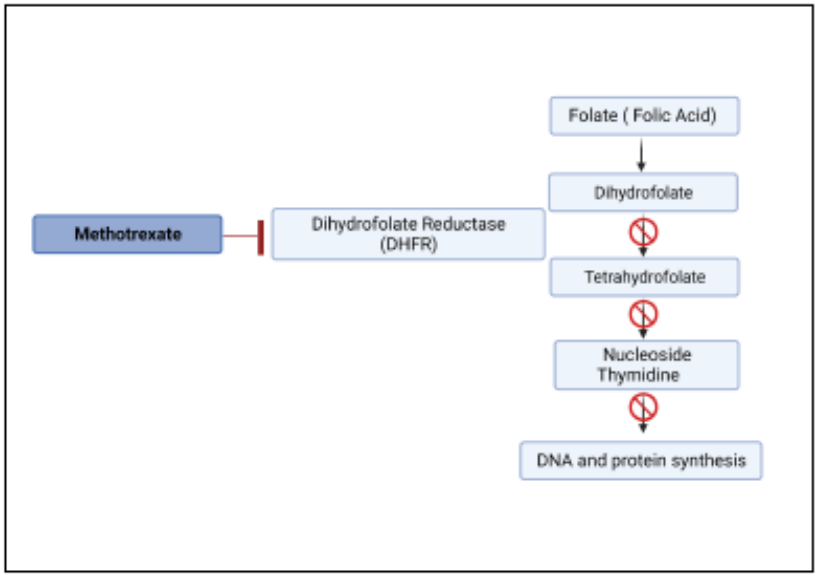

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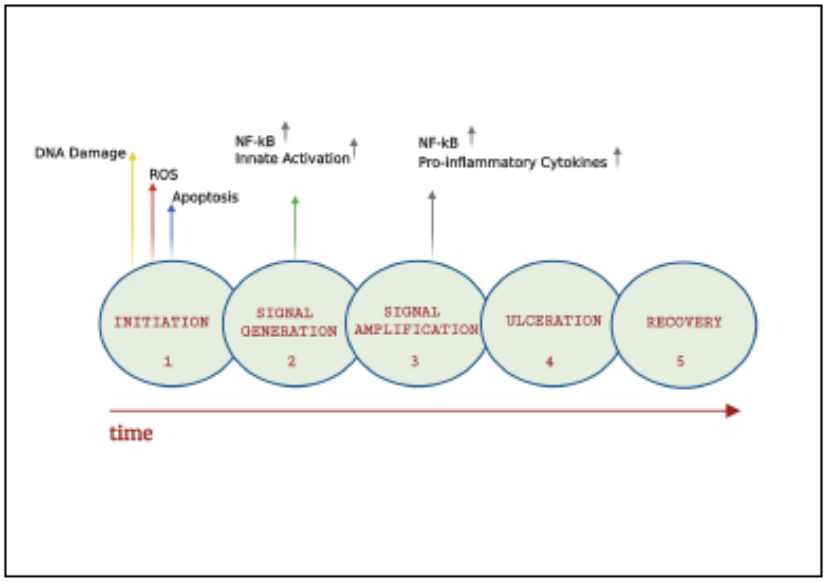

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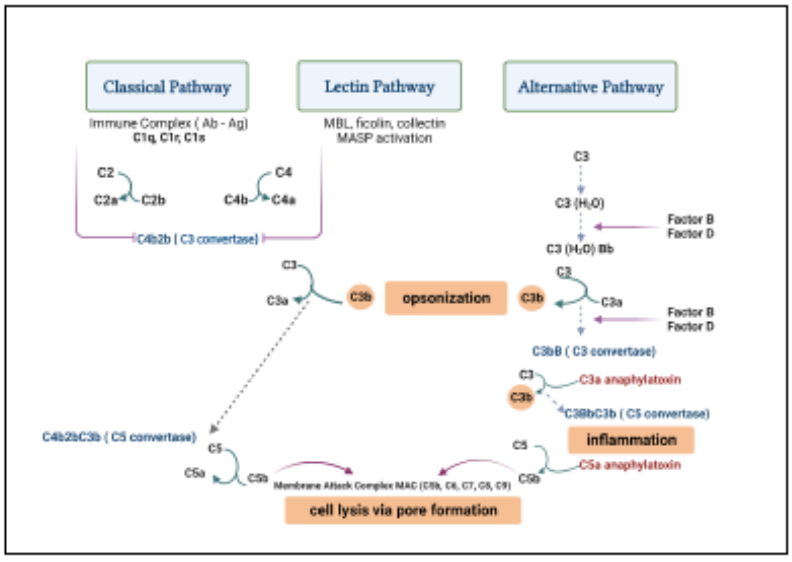

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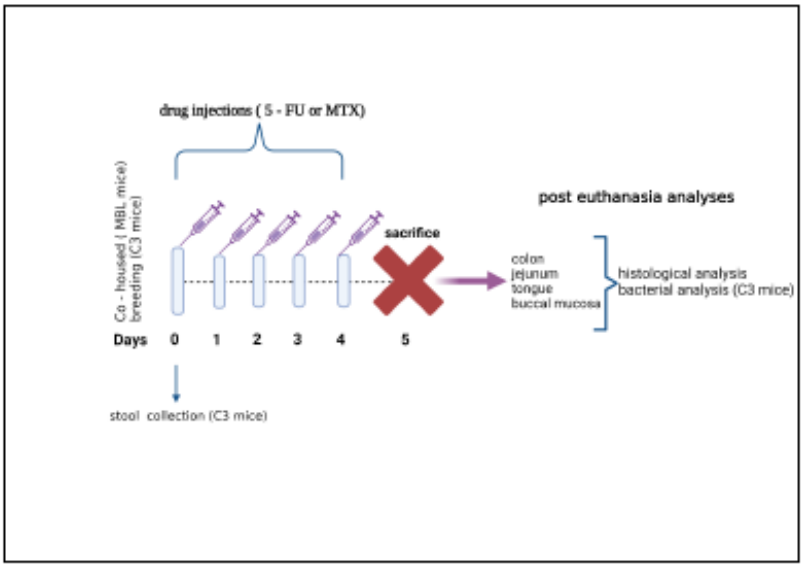

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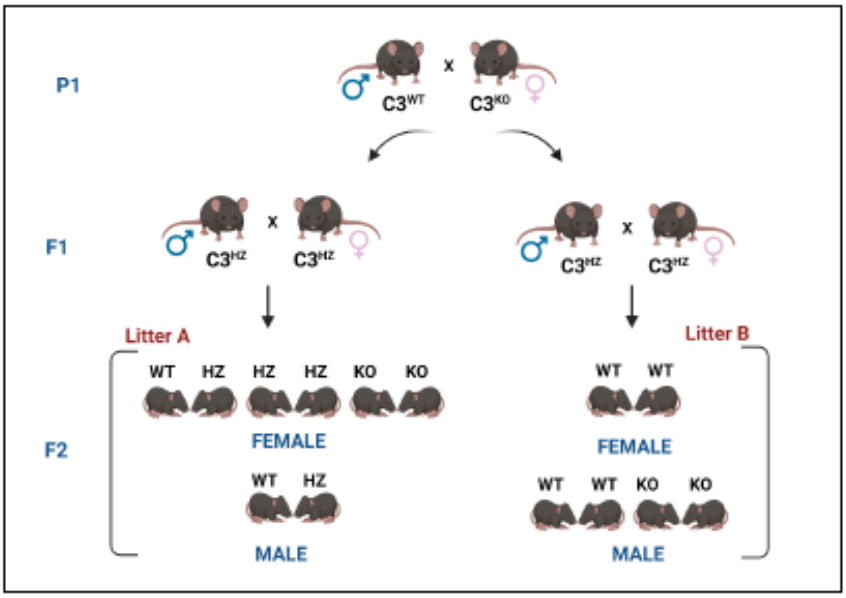

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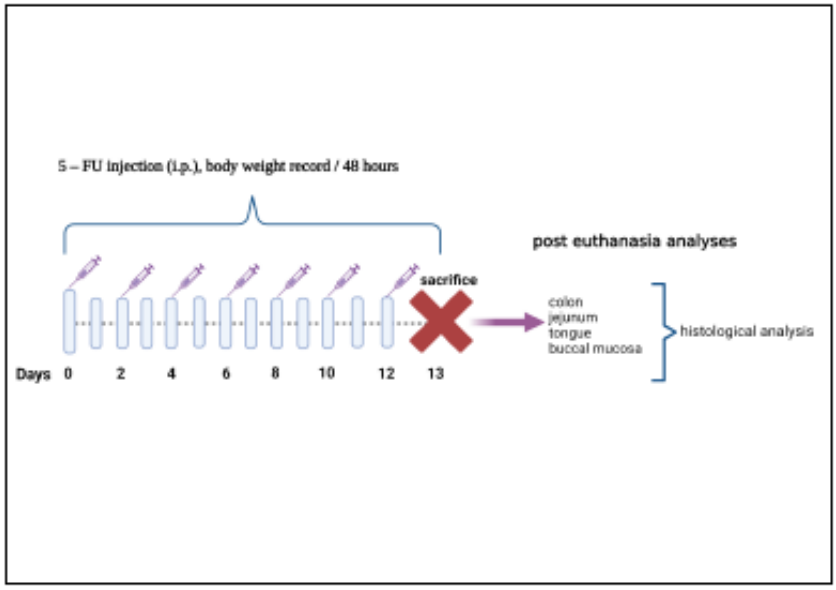

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