CHRONIC COLONIZATION OF *CLOSTRIDIOIDES DIFFICILE* FROM HUMAN COLON CANCER-ASSOCIATED BIOFILMS INDUCES COLON TUMORIGENESIS IN *APC*$^{MIN/+}$ MICE

by
Jie Chen

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland
June 2021

©2021 Jie Chen
All rights reserved

Abstract

Despite extensive studies on the pathogenesis of acute *Clostridioides difficile* (*C. difficile*) infection (CDI), CDI has never been associated with colorectal cancer (CRC). Analyzing microbial species sequences from our previous study, we identified *C. difficile* DNA in the subsets of biofilm-positive (BF+) human colonic mucosal homogenates that promoted colon tumorigenesis after inoculation into germ-free (GF) $Apc^{Min\Delta850/+}$;$Il10^{-/-}$ and $Apc^{Min\Delta850/+}$ mice or specific-pathogen-free (SPF) $Apc^{Min\Delta716/+}$ mice. These results raised an unexpected question of whether persistent mucosal colonization of *C. difficile* contributes to human colon tumor development. To address this question, we developed a chronic *C. difficile* infection mouse model in this study that allows sustained and non-lethal *C. difficile* colonization for 12 weeks in SPF $Apc^{Min\Delta716/+}$ mice. Our results show that toxin-producing *C. difficile* strains, especially our human CRC-associated isolate CI_m_2663, enhance colon tumor formation in SPF $Apc^{Min\Delta716/+}$ mice. In addition, we evaluated the spatial localization of *C. difficile* strains in the infected mouse colons. We detected most of *C. difficile* in the colon lumen with sparse mucus invasion, but *C. difficile* did not aggregate to assemble biofilms in either GF wild-type mice or SPF $Apc^{Min\Delta716/+}$ mice. The persistent *in vivo* production of *C. difficile* toxins, particularly toxin B, correlates with mouse colon tumor counts, suggesting that a toxin-dependent mechanism contributes to *C. difficile*-induced colon tumorigenesis. Of note, the colonization of toxigenic *C. difficile* strains induces low-grade chronic inflammation, displaying predominant infiltration of macrophages over time in colonic mucosa. Although pro-oncogenic cytokine IL-17A is similarly upregulated in all tumors regardless of *C. difficile* infection, toxigenic *C. difficile* strains suppress potential anti-tumorigenic cytokines IFN-γ

and IL-25 in tumors, suggesting *C. difficile* infection may modify tumor microenvironment, at least partly, by shaping inflammatory signatures. Our results support that chronic *C. difficile* toxin exposure predisposes normal colonic epithelial cells to a pro-inflammatory, pro-tumorigenic environment that fosters persistent epithelial hyperplasia and sequential tumor progression. In summary, toxigenic *C. difficile* chronic colonization enhances colon tumorigenesis in *Apc*$^{Min\Delta716/+}$ mice involving a mechanism dependent on persistent toxin B production and host immune responses.

Thesis Advisor and Primary Reader: Cynthia L. Sears

Co-Advisor: Jay H. Bream

Readers: Jay H. Bream**,** Fengyi Wan, Patricia J. Simner

Acknowledgment

First and foremost, all my praise and glory is humbly conveyed to our Almighty God for his love when I am hungry, his words when I stay foolish, and his guidance when I need indeed. Without His blessings and wisdom, it was not even possible for me to start my Ph.D. journey, to make any progress in the science He has granted, or to accept so many miracles in my life during the past 6 years.

It is with my deepest respect and humility to take this opportunity to show my gratitude to all who have encouraged and supported me in the journey of completing my thesis. It would be impossible to list all names in this brief letter, but I would give my special and sincere thanks to a few people.

First of all, my greatest thanks go to Professor Cynthia Sears sincerely for providing me with invaluable opportunities and many suggestions to conduct this research under your supervision. Your expert, knowledge, and endless support shaped me how to face challenges positively and how to think critically throughout my Ph.D. study.

I would like to express my exceptional appreciation to Dr. Jay Bream, Dr. Fengyi Wan, Dr. Patricia Simner, and Dr. Franck Housseau for being willing to serve on my thesis advisory committee with their expertise, encouragement and guidance, and standing with me through the ups and downs. My sincere thanks to Dr. Shaoguang Wu for her instrumental help and advice at each step in advancing my research. A great thank you to Dr. Nick Markham for being an amazing collaborator providing us with isogenic *C. difficile* strains and sharing his vast knowledge, brilliant ideas and expert experience.

Next, I am grateful wholeheartedly to all my lab mates in the Sears lab for supporting my work and their excellent cooperation. Particularly, I deeply appreciate the great efforts from Madison McMann, Dr. Xinqun Wu, Dr. Reese Knippel, Dr. Julia Drewes, Dr. Fyza Shaikh, Courtney Stevens and many others on helping me conduct lots of experiments. Without their excellent skills and strong work ethic, it would be impossible for me finish my thesis research within this time frame during the COVID-19 pandemic. A highest honor goes to the original initiators Dr. Christine Dejea and Dr. James White for picking *C. difficile* out of hundreds of bacterial species, and to the two summer students 2016 in our lab, Aishwarya Shettigar and Rebecca Gellman, for their hard working on isolating our CRC-associated *C. difficile* isolate.

I feel deeply thankful and blessed for the opportunity to study in a dedicated environment in W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health. All faculty and colleagues have shown their continuous support and inspiration that are necessary for us to work in a pandemic style and to achieve many life and research milestones.

I would like to thank all my families and friends for expressly inspiring me and cheering me up along the path. Especially, my profound gratitude goes to my husband Dan and my two sons Jayden and Benjamin for their unconditional love and endless joyful

moments. I never say enough thanks to my beloved parents for sharing my burden and making limitless sacrifice to endorse my dream. Nothing says love like being understood and supported by my parents. Last but not least, I would like to give my sincere and huge thanks to all my church families for sharing their faithful thoughts and keeping me in their prayers.

May God lead our research to the truth, integrity and elegance.
Love in Him.

Jie Chen
June 2021

Contents

Abstract ii

Acknowledgments iv

List of Tables x

List of Figures xi

List of Abbreviations xiii

Chapter 1 General Introduction and thesis objectives **1**

1.1 General introduction 1

1.2 Thesis hypothesis and objectives 4

1.3 Thesis approaches 4

1.4 Key discoveries 6

1.5 Study significance and clinical relevance 7

Chapter 2 Literature review **9**

2.1 Microbiota dysbiosis in human colon cancer: evidence of association and causality 9

2.1.1 Epidemiology and etiology of human CRC 9

2.1.2 Gut specific pathogens and CRC 10

2.1.3 Gut bacterial dysbiosis in CRC 11

2.1.4 Colonic biofilms in CRC 12

2.1.5 Colon-inhabiting oral microbes in CRC 14

2.2 *C. difficile* Microbiology 15

2.2.1 *C. difficile* chromosome 16

2.2.2 *C. difficile* life cycle 16

2.2.3 *C. difficile* cultivation and antibiotic resistance 18

2.3 Current epidemiology studies of CDI . . . 19
2.3.1 CDI clinical manifestation, risk factors, and antibiotic treatment . . . 19
2.3.2 Molecular epidemiology of CDI . . . 20
2.4 Pathogenesis of *C. difficile* . . . 21
2.4.1 *C. difficile* toxins . . . 21
2.4.2 Effects of *C. difficile* toxins on epithelial cells . . . 21
2.4.3 Binary toxin . . . 22
2.4.4 Non-toxin virulence factors . . . 23
2.5 Animal models of CDI and colon tumorigenesis in $Apc^{Min/+}$ mouse models . . . 26
2.5.1 Animal models of CDI . . . 26
2.5.2 Colon tumorigenesis in $Apc^{Min/+}$ mouse models . . . 27
2.6 *C. difficile* and human colon cancer . . . 28
Chapter 3 *C. difficile* strain isolated from human CRC tumor mucosa enhances tumor formation in chronically colonized SPF $Apc^{Min\Delta716/+}$ mice **31**
3.1 Abstract . . . 31
3.2 Introduction . . . 32
3.3 Materials and Methods . . . 35
3.3.1 DNA extraction and 16s rRNA amplicon sequencing . . . 35
3.3.2 *C. difficile* human strain isolation . . . 36
3.3.3 *C. difficile* strains and spore preparation . . . 37
3.3.4 Mouse breeding and housing . . . 38
3.3.5 Mouse experiment . . . 38
3.3.6 Colon harvest and tumor assessment . . . 40

3.3.7 Tissue RNA extraction, cDNA synthesis, and RT-qPCR 40

3.3.8 Fluorescence *in situ* hybridization (FISH) and confocal microscopy 41

3.3.9 Periodic Acid-Schiff (PAS) stain 42

3.3.10 Statistical Analysis 42

3.4 Results 42

3.4.1 Detection, isolation and characterization of a *C. difficile* isolate from human biofilm-positive tissue and subsequently inoculated mice 42

3.4.2 Establish chronic colonization of *C. difficile* in SPF $Apc^{Min\Delta716/+}$ mouse model 45

3.4.3 Toxigenic *C. difficile* strains enhance colon tumor formation in SPF $Apc^{Min\Delta716/+}$ mice 48

3.4.4 *C. difficile* colonized SPF $Apc^{Min\Delta716/+}$ mice show different morbidity and inflammatory features as compared with an acute colitis model using GF wild-type mice analyzed at two weeks after inoculation 50

3.4.5 Spatial localization of *C. difficile* in mouse colons and tumors 55

3.4.6 Phylogenetic analysis of *C. difficile* strains 60

3.5 Discussion 62

3.6 Conclusion 66

Chapter 4 Persistent *C. difficile* toxin production induces chronic mucosal inflammation and modifies epithelial biology that correlates with increased colon tumorigenesis in $Apc^{Min\Delta716/+}$ mice 67

4.1 Abstract 67

4.2 Introduction 67

4.3 Materials and Methods 72

4.3.1 *C. difficile* strains and mouse experiment 72

4.3.2 Cell Culture 73

4.3.3 Cytotoxicity Assay 73

4.3.4 Toxin B ELISA 74

4.3.5 Histopathologic assessment 75

4.3.6 Immunohistochemistry (IHC) staining 75

4.3.7 Tissue RNA extraction, cDNA synthesis, and RT-qPCR 77

4.3.8 Statistical Analysis 77

4.4 Results 78

4.4.1 Persistent *C. difficile* toxin production correlates with colon tumor counts 78

4.4.2 Persistent colonization with toxigenic *C. difficile* strains induces low-grade colonic inflammation from SPF $Apc^{Min\Delta 716/+}$ mice after 12-week colonization 82

4.4.3 Promotion of epithelial proliferation may be pro-tumorigenic when $Apc^{Min\Delta 716/+}$ mice are colonized chronically with toxigenic *C. difficile* strains 92

4.5 Discussion 95

4.6 Conclusion 99

Chapter 5 Summary **101**

5.1 General discussion 101

5.2 Conclusion 106

5.3 Future directions 106

Reference **108**

Curriculum Vitae **124**

List of Tables

Table 1. *C. difficile* primers and probes used in this study 37

Table 2. *C. difficile* validation with individual human mucosa from tumors and normal flanking regions 44

Table 3. Toxin genes and ribotypes of *C. difficile* strains used in this study 45

Table 4. Histopathological scoring criteria on mouse colon sections 75

Table 5. Gene List for the RT-qPCR analysis 76

Table 6. Qualitative fecal *C. difficile* toxin B ELISA 79

List of Figures

Figure 2.1 Spatial localization of the gut microbiota and bacterial biofilms in the colon 12

Figure 2.2 *C. difficile* life cycle and pathogenesis in humans 17

Figure 2.3 *C. difficile* toxin A and toxin B pathogenicity locus 21

Figure 3.1 *C. difficile* is detected persistently in human biofilm-positive mucosa and subsequently colonized germ-free mice that develop colon tumors 44

Figure 3.2 New antibiotic pretreatment facilitates *C. difficile* chronic colonization in SPF *Apc*$^{Min\Delta716/+}$ mice 47

Figure 3.3 Toxigenic *C. difficile* strains enhance colon tumor formation in *Apc*$^{Min\Delta716/+}$ mice at 12 weeks after inoculation 49

Figure 3.4 Morbidity and bacterial burden during/at 2 weeks after *C. difficile* inoculation in GF wild-type mice and SPF *Apc*$^{Min\Delta716/+}$ mice 51

Figure 3.5 Differential cytokine gene expression in the sub-acute phase at 2 weeks after *C. difficile* inoculation in GF wild-type mice and in SPF *Apc*$^{Min\Delta716/+}$ mice 53

Figure 3.6 *C. difficile* spatial localization in both outer and inner mucus layers in GF wild-type mouse colons at 2 weeks after inoculation 56

Figure 3.7 *C. difficile* spatial localization in SPF *Apc*$^{Min\Delta716/+}$ mouse colons at 2 weeks and 12 weeks after inoculation 57

Figure 3.8 Toxigenic *C. difficile* strains induce mucus production in mono-associated GF wild-type mice 59

Figure 3.9 Whole genome sequence comparison among *C. difficile* strains included in this study 61

Figure 4.1 Persistent toxin production for 4 weeks correlates with colon tumor incidence

. . . . 79

Figure 4.2 Toxigenic *C. difficile* strain 630Δ*erm* without persistent toxin B production fails to promote tumors in $Apc^{Min\Delta 716/+}$ mice 81

Figure 4.3 *C. difficile* chronic colonization induces low-grade chronic mucosal inflammation scattered in non-tumor colon epithelium that correlates with colon tumor formation in SPF $Apc^{Min\Delta 716/+}$ mice at 12 weeks after *C. difficile* colonization 83

Figure 4.4 Colonic cytokine profile in SPF $Apc^{Min\Delta 716/+}$ mice at 12 weeks after inoculation 85

Figure 4.5 Immune cell infiltration in colon tumors from $Apc^{Min\Delta 716/+}$ mice at 12 weeks after inoculation 87

Figure 4.6 Immune cell infiltration in non-tumor normal colon regions from $Apc^{Min\Delta 716/+}$ mice at 12 weeks after inoculation 88

Figure 4.7 CRC Patient 3728 tumor mucosa that contains toxigenic *C. difficile* CI_m_2663 induces a rapid recruitment of neutrophils but macrophages infiltrate the lamina propria over time in GF $Apc^{Min\Delta 716/+}$ mice 89

Figure 4.8 Low-grade chronic inflammation in non-tumor colon tissues is accompanied by epithelial cell proliferation at 2 weeks and 12 weeks after colonization 91

Figure 4.9 The expression of Wnt/β-catenin regulated genes in normal colons and tumors in SPF $Apc^{Min\Delta 716/+}$ mice at 12 weeks after inoculation 93

Figure 4.10 DNA damage assessment by γ-H_2AX staining in SPF $Apc^{Min\Delta 716/+}$ mice at 2 weeks and 12 weeks after inoculation 94

Figure 5.1 Schematic diagram of the mechanisms by which *C. difficile* enhances colon tumorigenesis in $Apc^{Min\Delta 716/+}$ mice 105

List of Abbreviations

C. difficile	*Clostridioides difficile*
ETBF	Enterotoxigenic *Bacteroides fragilis*
E. coli	*Escherichia coli*
F. nucleatum	*Fusobacterium nucleatum*
CRC	Colorectal Cancer
SPF	Specific Pathogen Free
GF	Germ Free
BF	Biofilm
RHO	Ras homolog
RAC	Ras-related C3 botulinum toxin substrate
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal-Regulated Kinase
MAPK	Mitogen-Activated Protein Kinase
Wnt	Wingless and Int-1
APC	Adenomatous Polyposis Coli
Min	Multiple intestinal neoplasia
IHC	Immunohistochemistry
FISH	Fluorescence In Situ Hybridization
PAS	Periodic acid–Schiff
qPCR	quantitative Polymerase Chain Reaction
ILCs	Innate Lymphoid Cells
DCs.	Dendritic Cells

CPE	Complete Cytopathic Effects
ELISA	Enzyme-Linked Immunosorbent Assay
Ocln	Occludin
Cldn3	Claudin 3
Cdh1	Cadherin 1/E-Cadherin
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
CCND1	Cyclin D1
Axin2	Axis inhibition protein 2
c-Myc	cellular Myelocytomatosis

Chapter 1

General Introduction and thesis objectives.

1.1 General introduction. The mammalian gut microbiota is a complex ecosystem that contains trillions of diverse microorganisms predominantly consisting of bacteria in addition to viruses and fungi (Bielanski and Haber 2020; Dominguez-Bello *et al*. 2019). The vast majority of these microbial communities inhabit the colon, segregated from the colonic epithelium by a protective mucus layer. These symbiotic or, sometimes, dysbiotic communities display tremendous interaction with the local mucosal environment and guide systemic responses. Thus the gut microbiota is proposed to play an integral role in the host's nutrition, immunity, metabolism, disease pathogenesis (Lloyd-Price *et al*. 2016; Proctor *et al*. 2019; The Integrative HMP (iHMP) Research Network Consortium 2016; Sears and Garrett 2014) and therapeutic efficacy (Matson *et al*. 2018; Coutzac *et al*. 2020).

Human colorectal cancer (CRC) is a multifactorial disease that develops under complex conditions involving genetic susceptibility, carcinogens, microbiota and chronic inflammation (Sears and Garrett 2014; Garrett 2019; Elinav *et al*. 2013). CRC risk factors such as high-fat diet, smoking, excessive alcohol consumption and obesity significantly modify the colonic microbiota (Brenner *et al*. 2014; Louis *et al*. 2014; Wu, G. D. *et al*. 2011) that may ultimately drive CRC development (Drewes *et al*. 2016; Garrett 2019; Chen, J. *et al*. 2017). Some single bacterial species, such as enterotoxigenic *Bacteroides fragilis* (ETBF), pks+ *Escherichia coli* (*E. coli*), and *Fusobacterium nucleatum* (*F. nucleatum*), have garnered attention for their presence in some subsets of human CRC, and their

tumorigenic potential in animal models (Wu, S. *et al*. 2009; Goodwin *et al*. 2011; Arthur *et al*. 2012; Bullman *et al*. 2017; Rubinstein *et al*. 2013). However, none of these bacteria are consistently found in all CRC cases, and many more microorganisms relevant to CRC likely remain to be identified. Much remains to be learned from translational studies of colon tumorigenesis using human fecal (Baxter *et al*. 2014; Wong *et al*. 2017; Li *et al*. 2019) or mucosal microbiota transferred to murine models (Tomkovich *et al*. 2019). Only recently, the emergence of next generation sequencing technologies, including high-resolution 16S rRNA gene sequencing and metagenomic technologies, has enabled us to appreciate the compelling discoveries of bacterial organization and functional activities associated with CRC.

Biofilms are classically defined as bacterial aggregates embedded in an extracellular polymeric matrix (Costerton *et al*. 2003; Swidsinski *et al*. 2005; Swidsinski *et al*. 2009; Welch *et al*. 2016). Colonic biofilms are comprised of dense aggregates of diverse bacterial species that invade the inner mucus layer (Swidsinski *et al*. 2005; Dejea *et al*. 2014). For decades, bacterial biofilms have been studied in the context of inflammatory bowel diseases where bacterial direct contact with colon epithelial cells likely perturbs epithelial function including the barrier properties of the colon epithelium (Swidsinski *et al*. 2009; Hall-Stoodley *et al*. 2004). In 2014, our lab first discovered that biofilms are associated with human CRC, predominantly on tumors located in the right or proximal colon extending to the hepatic flexure (Dejea *et al*. 2014). These observations extended discussions on how the CRC microbiome impacted CRC biology to a new area. Our studies have demonstrated that biofilm-positive (BF+) human colonic mucosa

homogenates, either from tumors or non-tumor tissues, reassembled biofilms and induced colonic tumors in both germ-free (GF) and specific-pathogen-free (SPF) *Apc*$^{Min/+}$ (Min: Multiple Intestinal Neoplasia) mice (Tomkovich *et al*. 2019). *Apc*$^{Min/+}$ mice has been long used as a murine model to study human CRC including hereditary CRC condition known as Familial Adenomatous Polyposis (FAP) that carries germline mutations in *APC*, and sporadic CRC among which somatic mutations in *APC* can be commonly found in about 80% colorectal tumors (Muzny *et al*. 2012; Rowan *et al*. 2000; Powell *et al*. 1992). Thus, as above, this mouse model can be used to test mechanisms that promote colon tumorigenesis and can help to identify novel bacterial species and virulence factors that contribute to colon tumor pathogenesis.

As will be shown in this thesis, 16S rRNA amplicon sequencing of human mucosal homogenates combined with detailed murine studies using GF *Apc*$^{Min\Delta850/+}$ and *Apc*$^{Min\Delta850/+}$;*Il10*$^{-/-}$ mice revealed that *Clostridioides difficile* (*C. difficile*), detectable in BF+ human mucosa homogenates, is remarkedly enriched in the subsequently inoculated GF mice, but absent from biofilm negative (BF-) human mucosa homogenates and associated mice. The persistent detection of *C. difficile* 16S rRNA gene sequence along with the colon tumor development in the inoculated GF mice raised a critical question whether toxigenic *C. difficile* plays an essential role in colon tumorigenesis. Although toxigenic *C. difficile* is a notorious intestinal pathogen that can result in extensive colitis (Chandrasekaran and Lacy 2017; Awad *et al*. 2015), *C. difficile* infection had not previously been linked to CRC. To date, no dedicated epidemiology studies have investigated the association between *C. difficile* infection and human CRC development

(Zheng *et al*. 2017; Chopra *et al*. 2010; Burgner *et al*. 1997), and similarly, very limited molecular studies have explored the controversial roles of *C. difficile* virulence factors in pro-tumorigenic signaling (Na *et al*. 2005; Tao *et al*. 2016; Chen, P. *et al*. 2018).

1.2 Thesis hypothesis and objectives. Given our intriguing discovery of *C. difficile* identification in mice that developed tumors after receiving human biofilm microbiota**,** we hypothesized that when persistently colonizing the colon, *C. difficile* promotes colon tumor formation and/or progression. If correct, this may suggest that a subset of individuals undergoing *C. difficile* infection-associated colitis or merely carrying *C. difficile* asymptomatically are at an increased risk of developing CRC over time. The mechanism(s) by which *C. difficile* contributes to the tumorigenic environment are unknown. Research is needed to improve understanding about *C. difficile* pathogenic effects in CRC. Herein, we hypothesized that, at least some, *C. difficile* strains could be pro-tumorigenic, acting through *C. difficile* virulence factors, host immune responses and/or microbial dysbiosis. To address the role of *C. difficile* in colon tumorigenesis, I sought to: (a) determine whether *C. difficile* alone is pro-tumorigenic in $Apc^{Min\Delta 716/+}$ mice and, if so, to characterize in detail the tumorigenesis; (b) identify whether *C. difficile* toxins contribute to colon tumorigenesis; (c) determine if *C. difficile* induces biofilm formation; and (d) assess *C. difficile*-induced mucosal immune responses that accompany or contribute to colon tumor development.

1.3 Thesis approaches. Given that CRC development from initiation to progression commonly takes more than 10 years in humans and that longitudinal microbiome

information from CRC patients is lacking, we decided to use the genetically tumor-susceptible mouse model, *Apc*$^{Min\Delta716/+}$ mice to test the capability of *C. difficile* to induce colon tumor formation. Since prior mouse models of *C. difficile* infection have focused on short-term, predominantly lethal models, our primary goal in this study was to create a murine model that allows the sustainable and non-lethal *C. difficile* colonization for 12 weeks to permit the testing of colon tumor development. First, we introduced a new antibiotic treatment combination to facilitate sustained *C. difficile* colonization in conventional SPF *Apc*$^{Min\Delta716/+}$ mice. Additionally, we optimized a qPCR assay to detect the tissue-associated *C. difficile* copy number from normal colon snips or tumors at 12 weeks post-inoculation. The localization of *C. difficile* in the colonic environment was visualized using Fluorescence *in situ* Hybridization (FISH) with a *C. difficile* species-specific probe.

Next, we aimed to determine whether *C. difficile* toxins (toxin A and toxin B) were associated with tumor incidence and/or enhanced Wnt/β-catenin signaling activities. To determine the role of *C. difficile* toxins in colon tumorigenesis, we utilized the non-toxigenic ATCC strain, toxin A-negative and toxin B-negative (A^-B^-), as a comparator with our toxigenic CRC-associated isolate. In parallel, we also inoculated other, non-CRC-associated toxigenic strains (A^+B^+ and A^+B^- mutant derivative) into *Apc*$^{Min\Delta716/+}$ mice with the purpose of assessing their capability to induce tumors and addressing whether tumorigenic potential is limited to the strain isolated from our BF+ CRC patient. To evaluate Wnt/β-catenin signaling activities, the classic tumorigenic signaling in *Apc*$^{Min\Delta716/+}$ mice, we measured Wnt downstream target gene expression.

Another important objective of this project was to profile the mucosal immune signatures induced by *C. difficile* chronic colonization and their roles in colon tumorigenesis. Although studies in diverse mouse models have characterized acute inflammatory components in *C. difficile* colitis, whether human *C. difficile* colonization in the colon provokes chronic inflammation is unknown. To investigate the inflammatory environment favoring *C. difficile*-driven tumorigenesis, the overall mucosal cytokine expression in non-tumor normal tissues or tumors were measured by qPCR. The immune cell infiltration in *C. difficile*-associated tumor and non-tumor tissues was evaluated by immune-histochemistry (IHC) targeting multiple immune cell populations.

1.4 Key discoveries. In this thesis project, we addressed the questions: (a) Is *C. difficile* one of the driver species that promote colon biofilm formation and/or tumorigenesis, or simply acting as a "passenger" in the tumor-related microbial dysbiosis? (b) Are *C. difficile* toxins and associated mucosal inflammation contributing to the tumorigenic mechanisms? In order to investigate the tumorigenic potential and related molecular mechanisms of *C. difficile* strains using $Apc^{Min\Delta716/+}$ mice, we successfully modified the antibiotic treatment from previously published recipes and established a chronic colonization model of *C. difficile* in SPF $Apc^{Min\Delta716/+}$ mice. We found that toxigenic *C. difficile* strains, particularly our human CRC-associated isolate, promote colon tumor incidence after 12 weeks in *C. difficile*-colonized SPF $Apc^{Min\Delta716/+}$ mice. The persistent production of *C. difficile* toxins appears to mediate tumor development, in part, by activating the classical Wnt/β-catenin signaling pathway but without inducing significant or sustained DNA damage. Although neither a *C. difficile* single-species biofilm nor a polymicrobial biofilm is recapitulated in

our mouse models, we demonstrated that *C. difficile* chronic colonization induces long-lasting colonic inflammation that correlates with tumor multiplicity. Therefore, this study demonstrated that toxigenic *C. difficile* promotes colon tumorigenesis in *Apc*$^{Min\Delta 716/+}$ mice through a mechanism likely dependent on long-term *C. difficile* colonization, persistent toxin production and host immune responses.

1.5 Study significance and clinical relevance

Both CRC and *C. difficile* infection are critical public health issues that have drawn extensive investigations in disease prevention, diagnosis and treatment. This project describes the association between *C. difficile* and the BF+ human CRC subset, and for the first time, tests the tumorigenic potential of *C. difficile* in a murine model.

CRC is the fourth most prevalent cancer worldwide in 2018 (Bray *et al*. 2018), and remains one of the leading causes of cancer-related deaths in both men and women despite a significant decline in incidence rates in the United States, particularly in people over age 50 years, due to colonoscopy screening (Brenner *et al*. 2014). Recent epidemiological data underscore an increasing CRC risk in the population younger than age 50 years (Siegel *et al*. 2017), and this disturbing trend may be associated with specific dietary factors and eating patterns that affect the gut microbiota. Therefore, the identification and characterization of new driver bacteria associated with CRC seems urgent and critical for advancing our knowledge of how the microbiota modifies CRC biology and to devise new approaches to CRC detection and prevention. With these objectives, we seek to identify new microbial features to guide colonoscopy screening

and the risk stratification for CRC. We thereby hope to contribute to improving clinical approaches of prevention, diagnosis, and even treatment of CRC.

Despite advances in diagnostic methods and technology, *C. difficile* infection (CDI) remains a critical health condition in human populations and difficult to cure in some patients. CDI has led to many major outbreaks over the past two decades (Warny *et al*. 2005; McDonald *et al*. 2005) due to, in particular, overuse of antibiotics and the emergence of *C. difficile* strains displaying antibiotic-resistance to overused antibiotics. *C. difficile* readily spreads with its production of highly disinfectant-resistant spores that foster CDI transmission. High CDI relapse rates of about 20% in CDI patients (Marsh *et al*. 2012; Lessa *et al*. 2015) and transmission through asymptomatic carriers (Kyne *et al*. 2002) present to be challenging clinical problems with CDI. Asymptomatic individuals contribute to transmission, whereas whether asymptomatic carriers are at higher risk of CDI remains unclear. Biofilm formation may promote latency and a reservoir for *C. difficile*. *C. difficile* biofilm formation may contribute to asymptomatic carriage with the potential to change epithelial biology and could be a reservoir for recurrent CDI. Herein, we sought to delineate the tumorigenic potential of *C. difficile* and its associated mechanisms using murine models, with which we hope to provide additional evidence assisting in stratifying patients considered for colonoscopy evaluation to advance colon cancer prevention.

Chapter 2

Literature review

2.1 Microbiota dysbiosis in human colon cancer: evidence of association and causality (Chen, J. *et al*. 2017)

2.1.1 Epidemiology and etiology of human CRC. Each year, over 1 million new cases of colorectal cancer (CRC) occur, which threaten 551,269 lives equally divided among men and women worldwide in 2018 (Bray *et al*. 2018). In the United States, excluding gender-specific cancers, CRC remains the second leading cause of cancer-related deaths in men and women despite a significant decline in incidence rates, particularly in people over age 50 years, due to colonoscopy screening (Bray *et al*. 2018; Brenner *et al*. 2014). Strikingly, recent epidemiological data demonstrate altered CRC incidence patterns in the United States from 1974 to 2013, notably underscoring an increasing CRC risk in the population younger than age 50 years (Siegel *et al*. 2017). This disturbing trend calls for new public awareness and research to delineate probable causes of age-specific CRC risk, particularly in the young.

CRC often takes more than 10 years to develop due to the accumulation of genetic mutations in colonic epithelial cells and sequential progression of the histopathological stages including aberrant crypt foci, polyps, adenomas and carcinomas (Fodde *et al*. 2001; Brenner *et al*. 2014). Hereditary forms of CRC only account for 3–5% of all cases, while more than 95% of CRC is sporadic and mainly occurs in people older than 50 years (Jasperson *et al*. 2010; Brenner *et al*. 2014). Besides genetic susceptibility, other risk

factors include inflammatory colitis, high consumption of red and processed meat, obesity, smoking and excessive alcohol consumption (Brenner *et al*. 2014; Louis *et al*. 2014; Wu, G. D. *et al*. 2011; Sonnenburg 2005). Strong evidence suggests these environmental and physiological factors significantly modify the colonic microbiota composition and function, which has led to increasing interest on the role of the microbiota in colon carcinogenesis (Garrett 2019; Goodman and Gardner 2018; Schwabe and Jobin 2013; Sears and Garrett 2014).

2.1.2 Gut specific pathogens and CRC. Based on our and others' studies using murine models, individual bacterial species provoke colon tumorigenesis by means of both microbial factors and host immune responses (Sears and Garrett 2014; Schwabe and Jobin 2013; Gagliani *et al*. 2014). Enterotoxigenic *Bacteroides fragilis* (ETBF) is common in the human microbiota and may be one of the more prevalent pathogens detected in human CRC patients. The mechanisms by which ETBF induce murine colon tumorigenesis include the induction of reactive oxygen species to directly initiate DNA damage, as well as activation of Wnt signaling pathways via E-cadherin cleavage by the *B. fragilis* toxin (BFT) and induction of proinflammatory cytokine IL-17A pathways to promote cell survival and proliferation (Wu, S. *et al*. 2009; Goodwin *et al*. 2011; Chung *et al*. 2018). Additionally, pks+ *Escherichia coli* (*E. coli*) (Arthur *et al*. 2012; Nougayrède *et al*. 2006) and superoxide-producing *Enterococcus fecalis* (Huycke *et al*. 2002) can putatively induce tumorigenesis by generating DNA mutagens. One recent study (Long *et al*. 2019) identified that *Peptostreptococcus anaerobius* (*P. anaerobius*), a gram-positive anaerobic bacterium, is enriched in the stools and mucosa of CRC patients, and

induced tumors in *Apc*$^{Min/+}$ mice. PI3K-Akt-NF-κB signaling mediates *P. anaerobius*-induced tumorigenesis by promoting cell proliferation and a pro-inflammatory microenvironment. Although the tumorigenic potential of these specific species has been studied in-depth with murine models, and each has been found in association with human CRC, we still lack an understanding of whether these bacteria or other microbes are causal in human CRC cases (Dejea *et al*. 2014; Kostic *et al*. 2013; Bullman *et al*. 2017; Garrett 2019).

2.1.3 Gut bacterial dysbiosis in CRC. In addition to the studies focusing on single pathogenic species, increasing studies have identified compositional shifts of the gut microbiota associated with CRC, which supports the hypothesis that altered microbial communities serve as contributors to CRC development (Chen, J. *et al*. 2017; Drewes *et al*. 2016; Goodman and Gardner 2018; Schwabe and Jobin 2013). For example, several studies demonstrate an emergence of putative pathogenic bacteria coincidently with substantial commensal depletion in CRC patients (Kostic *et al*. 2013; Dejea *et al*. 2014; Johnson *et al*. 2015; Tomkovich *et al*. 2019). The pro-carcinogenic mechanisms by which bacterial dysbiosis contributes to CRC are speculated to reflect microbial-induced alterations in host metabolism and mucosal immune responses (Sears and Garrett 2014; Louis *et al*. 2014). However, studies on the gut microbiota profiling in human CRC have yielded diverse results regarding the patterns of bacterial communities enriched in tumor regions as previously studied (Dejea *et al*. 2014; Drewes *et al*. 2017; Bullman *et al*. 2017; Tomkovich *et al*. 2019). It remains uncertain whether bacterial dysbiosis is merely secondary to or a causative exposure prior to the onset of carcinogenesis.

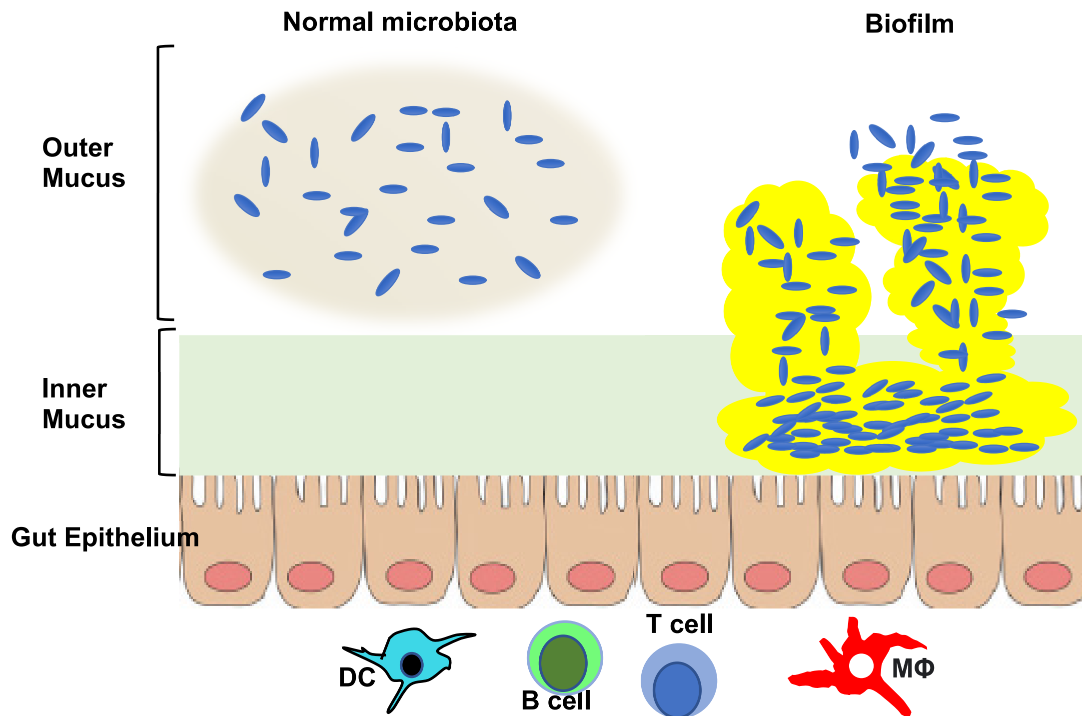


Figure 2.1 Spatial localization of the gut microbiota and bacterial biofilms in the colon. The inner mucus layer (light green) lining gut epithelium separates the luminal bacteria from the epithelial surface. Colonic biofilms are bacterial communities invasive into the inner mucus layer and in close proximity to epithelial cells and immune cells in lamina propria. DC: Dendritic Cell; MΦ: Macrophage.

2.1.4 Colonic biofilms in CRC. Bacterial biofilms are complex ecosystems composed of polymicrobial aggregates of diverse bacteria embedded in an extracellular polymeric matrix, mainly consisting of polysaccharides as well as proteins, nucleic acids and lipids (Hall-Stoodley *et al*. 2004). Colon biofilms are invasive into the dense inner mucus layer and directly interact with epithelial cells (Figure 2.1), which has been studied in inflammatory bowel disease for decades (Swidsinski *et al*. 2009). In 2014 our lab reported, for the first time, the association between biofilms and human CRC (Dejea *et al*. 2014), and defined that biofilms represent dense bacterial community invasion into the inner mucus layer spanning at least a linear distance of 200 μm across the colonic epithelial

surface (Swidsinski *et al*. 2005; Dejea *et al*. 2014). Colonic biofilms were a nearly universal feature of right-sided CRC and their paired normal mucosa obtained from the distant edge of surgical resections (Dejea *et al*. 2014). Interestingly, right colon cancers were not uniquely susceptible to biofilm formation. Approximately 17% (Drewes *et al*. 2017) of left colon cancers and 13% (15/120) of colonoscopy biopsies collected from healthy people at Johns Hopkins Hospital also displayed biofilms. In colonoscopy patients, often healthy individuals, the bacterial composition of these biofilms were typically less dense and thinner than the exuberant structures on tumors in CRC patients. The colonic epithelial cells under the biofilms in the paired normal tissues of CRC patients, or in normal colon biopsies obtained from healthy individuals, exhibited reduced or redistributed E-cadherin, enhanced IL-6 production, activated Stat3, and increased epithelial cell proliferation that are consistent with a pro-oncogenic state (Dejea *et al*. 2014).

Colonic biofilms associated with sporadic CRC are all polymicrobial entities (Dejea *et al*. 2014; Bullman *et al*. 2017; Tomkovich *et al*. 2019). Thus, defining the bacterial organization and function of biofilms from CRC patients at the species and/or strain level will provide new insights into mechanisms of microbiota-driven CRC. A collaborative study (Johnson *et al*. 2015) demonstrated that bacterial biofilms altered the cancer or normal colonic mucosal metabolome via upregulating polyamine metabolism, a possible promoter of colonic epithelial proliferation and cancer progression. Biofilm-associated enhancement of polyamine metabolites was associated with enriched Clostridial groups including *Sporobacter, Peptostreptococcaceae* and *Ceilonellaceae*, but reduced

Bacteroidales. Recent data from the Sears and Jobin laboratories support that bacterial communities in human colonic biofilms are pro-carcinogenic when transferred to mouse models (Tomkovich *et al*. 2019). However, it remains unclear whether some bacterial species play a more important role in leading biofilm formation and/or colon tumorigenesis, as versus the microbial community as a whole. A clear causal role for any bacterium, virulence factor or community structure in human CRC has yet to be well-defined. Prospective and longitudinal epidemiological studies are needed to determine the role of microbial communities in the initiation and progression of CRC.

2.1.5 Colon-inhabiting oral microbes in CRC. The finding of *Fusobacterium* in about 30% or more of CRC cases by 16S rRNA amplicon sequencing (Ahn *et al*. 2013; Kostic *et al*. 2012; Castellarin *et al*. 2012) was unexpected and has initiated discussions of an association between the oral microbiota and CRC, a topic expanded upon by other authors (Vogtmann and Goedert 2016; Flynn *et al*. 2016; Bullman *et al*. 2017). The *Fusobacterium* genus, particularly *Fusobacterium nucleatum* (*F. nucleatum*), is more frequently identified in CRC cases compared with less consistent results in colorectal adenoma cases, suggesting that *Fusobacterium* may contribute to later progression instead of earlier initiation of colon carcinogenesis (Ahn *et al*. 2013; Feng *et al*. 2015; Zackular *et al*. 2014; Bullman *et al*. 2017; Garrett 2019). Alternatively, increased, but modest, multiplicity of colon adenomas in susceptible mouse models in some, but not all experiments, may reflect a capacity of *F. nucleatum* for tumor initiation or promotion (Rubinstein *et al*. 2013; Yang *et al*. 2017; Kostic *et al*. 2013; Tomkovich *et al*. 2017). *F. nucleatum* appears to utilize several adherence factors such as FadA combined with a

proclivity to invade tissue, disrupt cell-cell adhesion, activate Wnt cell proliferation signals and stimulate pro-inflammatory pathways to potentially enhance the accumulation of genetic mutations or promote colon cancer biology (Rubinstein *et al*. 2013; Kostic *et al*. 2013; Yang *et al*. 2017). In addition to *Fusobacterium*, other groups of oral bacteria such as *Porphyromonas*, *Peptostreptococcus*, *Prevotella* and *Gemella* genera are often found associated with the colon microbiome of patients with colon cancer (Zackular *et al*. 2014). The carcinogenic potential and virulence factors of these genera are largely unknown. Furthermore, it remains to be validated whether these oral microbes detected in the colon represent the same species or strains that inhabit the oral cavity. Even if the oral bacteria residing in the colon originate from the oral cavity, we do not yet understand the mechanisms needed to adapt to the colonic environment and the implications in colon carcinogenesis.

2.2 *C. difficile* Microbiology. *C. difficile* is a gram-positive, obligate anaerobe that is capable of producing spores. The name *C. difficile* reflects its nature and the difficulty with isolation and microbial culture of this microbe. It belongs to the class of *Clostridia*, the family of Clostridiaceae, and the phylum of Firmicutes, and was formerly known as *Clostridium difficile* or *Peptoclostridium difficile* in *Clostridium* cluster XI (Ohashi and Fujisawa 2019; Galperin *et al*. 2016; Sandhu and McBride 2018). In 2016, *C. difficile* species was reassigned from *Clostridium* to the new genus *Clostridioides*, thus giving it the name *Clostridioides difficile* (Lawson *et al*. 2016). *C. mangenotii* is the only other species in this new genus.

2.2.1 *C. difficile* chromosome. The *C. difficile* genome exhibits genetic and phenotypic diversity consisting of a single circular chromosome of approximately 4.3 Mbp and a plasmid of 7-8 kbp (Sebaihia *et al*. 2006). Its genome shows only 15% homologous sequences with four other clostridial genomes including *C. acetobutylicum*, *C. botulinum*, *C. perfringens* and *C. tetani*, whereas, 50% of sequences are unique to *C. difficile* species (Sebaihia *et al*. 2006). PCR-ribotyping is one of the common methods used to demonstrate the molecular epidemiology of *C. difficile* strains based on 16S-23S rDNA intergenic spacer region patterns. Other molecular typing methods (Killgore *et al*. 2008) for strain characterization include toxinotyping (*tcdA*/*tcdB* sequence polymorphisms), pulse-field typing and restriction endonuclease analysis typing (whole genome restriction pattern polymorphisms), MLST and MLVA (multi-locus repeat- or non-repeat-based sequence variations), and SLP typing (surface-protein variations).

2.2.2 *C. difficile* life cycle. The *C. difficile* life cycle (Britton and Young 2014) includes dynamic changes and genetic regulation during the transformation between two forms of this microorganism, spores and vegetative cells (3). *C. difficile* spores are the primary transmission form that survive for long periods with high resistance to heat, aerobic and harsh circumstances. The spores inhabit a natural reservoir of soil and feces of domestic animals and humans, and any surfaces contaminated with feces. In contrast, *C. difficile* vegetative cells are the outgrowth form with metabolic activity and unable to be sustained outside of the host due to sensitivity to an aerobic environment. Once spores are ingested, their acid resistance protects their viability from the strong acidic conditions in the stomach, and the spores pass through to the colon where they can germinate into

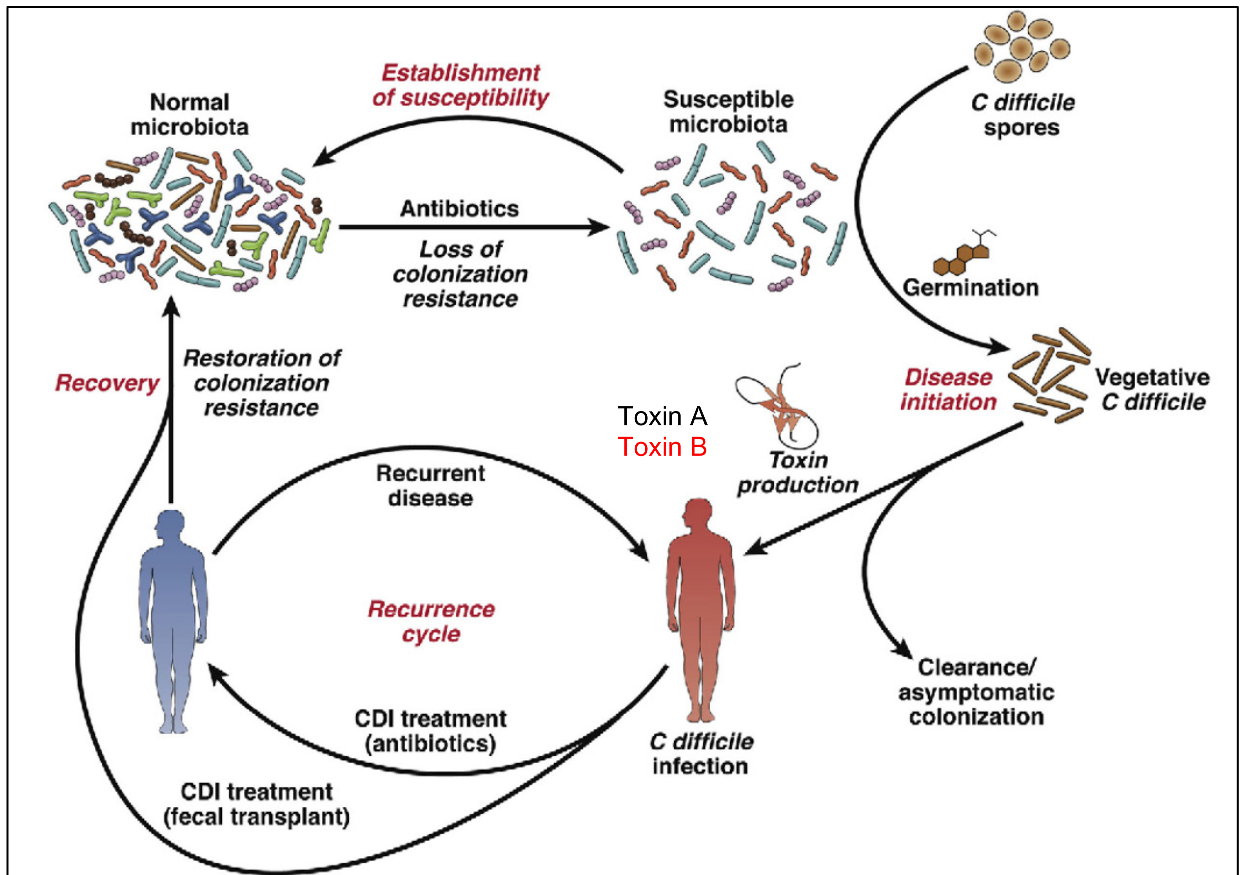


Figure 2.2 *C. difficile* life cycle and pathogenesis in humans. (Adapted from Britton R. A. *et al*. 2014)

vegetative cells in the presence of primary bile acids (Sorg and Sonenshein 2008; Howerton *et al*. 2011; Wilson 1983). The primary bile acids, mainly consisting of cholic and chenodeoxycholic acid, are synthesized in the liver, conjugated with taurine or glycine, and pass through to the colon (Jones *et al*. 2008; Chiang 2004). The colonic microbiota metabolizes primary bile acids to secondary bile acids, such as lithocholic and deoxycholic, that are inhibitory for *C. difficile* spore germination (Britton and Young 2014; Sorg and Sonenshein 2009), thereby suppressing *C. difficile* colonization. Only when the microbial balance is disrupted, mainly by the introduction of antibiotics (Britton and Young 2014; Buffie *et al*. 2012; Theriot *et al*. 2011; Rupnik *et al*. 2009), does the increased

availability of favorable nutrients including primary bile acids, amino acids, and carbohydrates (Sorg and Sonenshein 2008; Theriot *et al*. 2014), support *C. difficile* germination, proliferation and toxin production, leading to epithelial damage and inflammation (Rupnik *et al*. 2009; Kachrimanidou and Tsintarakis 2020; Zhu *et al*. 2018).

2.2.3 *C. difficile* cultivation and antibiotic resistance. *In vitro* bacterial culture studies revealed 100- to 1000-fold increased *C. difficile* germination rates when using BHI agar plates supplemented with the primary bile acid derivatives such as cholate, taurocholate, and glycocholate as vs BHI agar plates alone (Lawley *et al*. 2009). This observation allowed researchers to recover and isolate *C. difficile* strains from clinical and environmental samples (Karen and John 2011). This knowledge is also useful to quantify the *C. difficile* fecal bacterial burden in animal model studies. Environmental spores infect mice in a dose-dependent manner and about 7 spores per cm^2, which is about 11 spores per mouse colon, are needed to infect 50% of the mice (ICD50) (Lawley *et al*. 2009; Karen and John 2011), however, this may vary in a strain-dependent manner. Although *C. difficile* spores are resistant to high temperatures, 70% ethanol and antimicrobials, sporicidal agents such as bleach and benzyl chloride can successfully inactivate spores and are widely used in laboratory and clinical settings for decontamination. Furthermore, *C. difficile* vegetative cells intrinsically display no response *in vitro* to multiple antibiotics, such as aminoglycosides, tetracyclines, erythromycin, clindamycin, cephalosporins, and fluoroquinolones; in addition, clindamycin, cephalosporins, and fluoroquinolones are most commonly associated with increased risk of CDI (McDonald *et al*. 2018; Kelly and LaMont 2008; Song and Kim 2019; Marsh *et al*. 2012; Sebaihia *et al*. 2006).

2.3 Current epidemiology studies of CDI

2.3.1 CDI clinical manifestation, risk factors, and antibiotic treatment. *C. difficile* was first isolated in 1935 from a healthy child (HALL and O'Toole 1935), but was not identified to cause antibiotic-associated diarrhea and pseudomembranous colitis until 1978 (1978). *C. difficile*-associated diseases range from mild and self-limiting, watery diarrhea to severe bloody diarrhea, toxic megacolon or pseudomembranous colitis, that can be fatal (Britton and Young 2014). The risk factors for *C. difficile* infection include antibiotic exposure, advanced age, the use of gastric acid-suppressing drugs, hospitalization and host variables like immunodeficiency. Following exposure most often to antibiotics, the disrupted fecal microbial composition displays reduced microbiota diversity that contributes to the development of CDI (Kachrimanidou and Tsintarakis 2020). In 2011, *C. difficile* infection occurred causing approximately 453,000 cases and threatened 29,000 lives in the United States (Lessa *et al*. 2015; Guh *et al*. 2020). Further, the high recurrence rate of CDI after therapy increases the complexity of management and difficulties in disease control. Epidemiology studies show that about 20-30% of patients that receive CDI antibiotic treatment with metronidazole or oral vancomycin experience a recurrence after the treatment is discontinued (Marsh *et al*. 2012; Kelly and LaMont 2008; Song and Kim 2019). While the antibiotic treatment of CDI often cures CDI, the collateral microbiota 'damage' can result in ongoing dysbiosis that renders the host highly susceptible to reinfection or relapse. Fidaxomicin, recently added as a first-line CDI therapy (McDonald *et al*. 2018; Guery *et al*. 2018), is a non-absorbable antibiotic that is less toxic to obligate anaerobic commensal bacteria and reduces recurrence for *C. difficile* strains other than ribotype 027 (Abt *et al*. 2016; Louie *et al*. 2011).

2.3.2 Molecular epidemiology of CDI. With the spread of *C. difficile* infections worldwide during the past two decades, toxigenic *C. difficile* strains, producing toxin A (gene *tcdA*) and/or toxin B (gene *tcdB*), continue to be isolated from human hosts (Warny *et al*. 2005; McDonald *et al*. 2005; Loo *et al*. 2005). As previously discussed, multiple molecular typing methods (Killgore *et al*. 2008; Vedantam *et al*. 2012) are commonly used for strain identification, including PCR-ribotyping, pulsed-field gel electrophoresis (PFGE), restriction endonuclease analysis (REA) of total DNA, and toxinotype. For example, the predominant *C. difficile* strain isolated from the recent CDI epidemic outbreaks in the United States, Canada, UK and other European countries, and Asia was strain 027/NAP1/BI, referred to as ribotype 027, North American pulsed-field type 1 (NAP1), and group BI by restriction endonuclease analysis (Voelker 2010). 027/NAP1/BI is a hypervirulent strain with toxinotype III that produces higher quantities of toxin A and toxin B *in vitro* and appears to show increased virulence, particularly in outbreak settings. Some other well-characterized *C. difficile* strains prevalent in humans consist of 001/NAP2/J, 017/NAP9/CF, 078/NAP7/BK , and 106/NAP11/DH (Voelker 2010); all these strains are *tcdA* and *tcdB* positive except 017/NAP9/CF that is a toxin A^-, toxin B^+ strain. Ribotyping surveillance data has shown that the molecular epidemiology of the disease is shifting. The prevalence of the epidemic ribotype 027 is decreasing globally, while ribotypes 106 and 017 have become the most common strains in Europe and Asia, respectively (Vedantam *et al*. 2012). The most recent national data provided by the Centers for Disease and Control (CDC) shows similar trends in the US, using data derived from 1000 to 1500 annual samples submitted from ten states, not including Texas (Guh *et al*. 2020).

2.4 Pathogenesis of *C. difficile*

2.4.1 *C. difficile* toxins. *C. difficile* produces two major large clostridial toxins (LCTs), toxin A and toxin B. Both toxins are recognized as *C. difficile* virulence factors and likely contribute to *C. difficile*-associated disease (Vedantam *et al*. 2012; Kuehne *et al*. 2010; Chandrasekaran and Lacy 2017). However, strains that produce only toxin B are fully virulent in humans; no strains only secreting toxin A are known to cause human disease. This observation underpins the use of toxin B detection alone for CDI diagnosis. These two large secreted proteins are glucosyltransferases containing four structurally homologous domains and have a molecular mass of 308 kDa and 269.6 kDa, respectively (Karen and John 2011). The genes encoding toxin A and toxin B, *tcdA* and *tcdB*, are clustered with three accessory genes *tcdR*, *tcdC*, and *tcdE* and located within the 19.6 kb pathogenicity locus (PaLoc) (Figure 2.3) (Karen and John 2011). While *tcdR* and *tcdC* are negative and positive regulators of toxin production respectively, *tcdE* encodes a putative holin and facilitates toxin secretion.

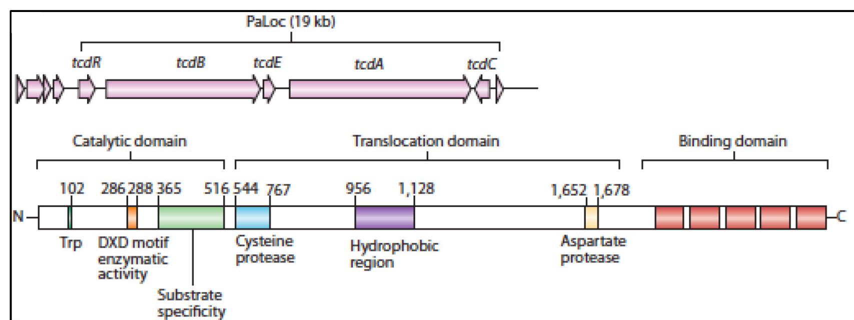


Figure 2.3 *C. difficile* toxin A and toxin B pathogenicity locus (Karen and John 2011)**.**

2.4.2 Effects of *C. difficile* toxins on epithelial cells. The direct effects toxin A and toxin B display on epithelial cells can be classified as glucosylation-dependent or -independent effects. The glucosylation-dependent effects include cytopathic effects that

cause cell skeleton disruption and the loss of cell-cell tight junctions, and cytotoxic effects comprise cell programmed death, apoptosis (Chandrasekaran and Lacy 2017; Kuehne *et al*. 2010). *C. difficile* induces apoptosis either through the death receptor-dependent extrinsic pathway triggered by toxin A and toxin B or through the mitochondria-dependent intrinsic pathway particularly when toxin B is at a lower concentration (Chumbler *et al*. 2016). While at higher concentrations (100 pM or above), toxin B initiates the assembly of the NADPH oxidase (NOX) complex on endosomes and aberrant production of endosomal reactive oxygen species (ROS) (Farrow *et al*. 2013; Wohlan *et al*. 2014; Chumbler *et al*. 2016), hereby inducing epithelial necrosis (glucosylation-independent cytotoxic effects). The mechanisms operative *in vivo* are still not clear, but it has been proposed that high levels of ROS may promote cellular necrosis through DNA damage, lipid peroxidation, protein oxidation and/or mitochondrial dysfunction (Temple *et al*. 2005; Daiber 2010; Prior *et al*. 2016). It has been suggested that, unlike toxin B, toxin A does not enhance ROS production, but causes a glucosylation-dependent apoptosis at all concentrations (Chumbler *et al*. 2016). Indirect effects of toxin A and toxin B on epithelial cells have been demonstrated to be mediated by inflammatory factors including cytokines, chemokines and reactive oxygen mediators from innate and adaptive immune cells (see details in Chapter 3).

2.4.3 Binary toxin. Besides toxin A and toxin B, some *C. difficile* strains produce a third toxin called binary toxin that is termed the *C. difficile* transferase (CDT) capable of catalyzing ADP-ribosylation in host cells. Binary toxin is composed of two unlinked proteins, CdtB and CdtA, and encoded on Cdt locus (CdtLoc) separate from the PaLoc

(Karen and John 2011). CdtA is the enzymatic component and CdtB has a binding function to transport CdtA into target cells (Gerding *et al*. 2014). These two components together cause cytotoxicity leading to cytoskeleton disruption, excessive fluid loss, cell rounding, and eventual cell death (Gerding *et al*. 2014; Cowardin *et al*. 2016; Berry *et al*. 2017). Although highly virulent and epidemic *C. difficile* strains, such as 027, 078 and CD196, also produce binary toxin in addition to toxin A and toxin B (Awad *et al*. 2015; Metcalf and Scott Weese 2011; Viswanathan *et al*. 2010), the role of binary toxin in *C. difficile* pathogenicity remains unclear. Some *in vitro* studies suggest that binary toxin may promote *C. difficile* adherence to Caco-2 cells using cell culture (Schwan *et al*. 2009). However, Metcalf and Weese showed that the biological activities of binary toxin are largely unknown *in vivo* and *C. difficile* strain production of binary toxin was not associated with the severity of *C. difficile* infection (Metcalf and Scott Weese 2011). Other case control studies showed that patients infected with binary toxin-positive strains tended to exhibit a higher mortality rate (Bacci *et al*. 2011), whereas no studies address whether binary toxin is causal to increased mortality.

2.4.4 Non-toxin virulence factors. In addition to *C. difficile* toxins, non-toxin virulence factors are increasingly emphasized given potentially important roles in colonization and persistence as well as organism proliferation and toxin regulation that are closely associated with *C. difficile* disease pathogenesis. These virulence determinants could contribute to the high recurrence rates observed in CDI (e.g., up to 33% in patients infected with the recent epidemic strain 027 after a first CDI episode, and 45% after a second episode) (Vedantam *et al*. 2012). Here we provide a current understanding about

the non-toxin virulence factors involved in sporulation, flagella, surface proteins, biofilm formation and quorum sensing.

Sporulation. Admittedly, spores, as a metabolically dormant form, are not able to produce toxins or directly cause epithelial damage leading to disease. A few epidemic studies have suggested that the *C. difficile* strains producing more and resilient spores, such as the 027 strain, tend to cause severe disease in CDI patients (Awad *et al*. 2015; Viswanathan *et al*. 2010). In other words, an increased ability to form spores in the host might be associated with higher virulence. However, the efficiency of sporulation *in vivo*, and its contribution to the prevalence of specific *C. difficile* strains and disease severity, is still an open question. The downstream regulators, and signaling molecules of Spo0A (Vedantam *et al*. 2012), a master regulator of sporulation, are highly conserved in *Bacillus spp.* and clostridia, however, the upstream factors influencing the sporulation checkpoint and the corresponding signaling pathways remain to be studied for clostridia.

Flagella. Flagella are filamentous organelles that drive cell movement and can play a significant role in some bacterial species for host invasion and colonization. For instance, *Helicobacter pylori* flagella are critical to its adherence and colonization (Bergonzelli *et al*. 2006; Dunne *et al*. 2014). Likewise, flagella allow *C. difficile* to be motile and pass through the mucus layer to initiate adherence to the epithelial cells (Stevenson *et al*. 2015; Tasteyre *et al*. 2001). FliC, the major flagellin structural monomer, and FliD, the cap protein are two major *C. difficile* flagellar proteins that have been well characterized (Tasteyre *et al*. 2001). Not all pathogenic *C. difficile* strains show a flagellated phenotype by electron microscopy, but the flagellin gene universally exists in the *C. difficile* genome

(Tasteyre *et al*. 2000). The role of flagella in CDI pathogenesis is contentious and appears to be strain dependent. Studies with stain R20291 (ribotype 027) showed that *C. difficile* flagella promoted adherence to cells and intestinal tissue (Baban *et al*. 2013), whereas, absence of flagella in strain 630Δerm did not reduce adherence (Tasteyre *et al*. 2001; Baban *et al*. 2013).

Surface proteins. S-layers are surface-exposed proteinaceous layers coating the bacterial cell surface. S-layer proteins (SLPs) are the most well-characterized family of *C. difficile* cell wall proteins (CWPs). SLPs and CWPs have been proposed as putative virulence factors and may play a role in *C. difficile* adherence and colonization on the colon epithelium. *C. difficile* SLPs are derived from the polypeptide precursor, SlpA, by proteolytic cleavage by the protease Cwp84 (Dang *et al*. 2010). Cwp84 is the best studied cell wall protein and a cysteine protease involved in processing the S-layer (la Riva, de *et al*. 2011). Cwp84 has also been shown to degrade extracellular matrix proteins *in vitro*, suggesting that this protein may play a role in tissue degradation and bacterial dissemination during infection (Janoir *et al*. 2007).

Biofilm formation and quorum sensing. Evidence regarding *C. difficile* biofilm formation remains insufficient as studies only suggest *C. difficile* may form biofilms *in vitro* but not *in vivo*. Nevertheless, key questions remain regarding the mechanisms by which *C. difficile* accomplish biofilm assembly and whether biofilms enhance *C. difficile* persistence and virulence in hosts. *C. difficile* R20291 was demonstrated to yield more biofilms *in vitro* compared to *C. difficile* 630 (Dapa and Unnikrishnan 2013), suggesting

C. difficile capability for biofilm formation varies between strains. Cwp84 and LuxS are virulence-associated proteins that promote *C. difficile* biofilm formation (Soavelomandroso *et al*. 2017; Dapa and Unnikrishnan 2013; Dapa *et al*. 2013). Quorum sensing, a form of bacterial cell-cell communication, plays a key role in *C. difficile* biofilm formation in early and late stationary phases when the bacteria start to produce extracellular matrix composed of proteins, polysaccharide and DNA. In this process, the LuxS protein is a putative regulator affecting both biofilm assembly and biological activities within the biofilm microbial community (Dapa and Unnikrishnan 2013).

2.5 Animal models of CDI and colon tumorigenesis in *Apc*$^{Min/+}$ mouse model

2.5.1 Animal models of CDI. Various animal models have been developed to study *C. difficile* infection and disease pathogenesis, such as hamster, mouse, rat, rabbit, infant pig and so on (Hutton *et al*. 2014; Best *et al*. 2012; Lawley and Young 2013). Until recently, hamsters were most commonly used for modeling *C. difficile* infection. The hamster model has an integral role in studying *C. difficile* pathogenesis (Best *et al*. 2012) as pathological signs observed in a hamster after infection mirror many clinical manifestation of human CDI (Bartlett *et al*. 1977; Price *et al*. 1979). Hamsters were employed as important tools to assess the role of *C. difficile* toxins by comparing the infection severity of isogenic *C. difficile* mutants (Lyras *et al*. 2009; Kuehne *et al*. 2010; Kuehne *et al*. 2014). However, murine models have become increasingly popular and important in unravelling diverse aspects of CDI including colonization, host susceptibility, disease pathophysiology and recurrence (Hutton *et al*. 2014; Lawley and Young 2013). Mouse models of CDI have been continuously improving and are now extensively used because

infection susceptibility, similar to humans, can be induced by various antibiotics and mouse-specific reagents allowed better analysis of host tissues. Similar to human CDI, mouse susceptibility must be induced by disrupting the microbiota through antibiotic treatment. The effect of antibiotics on the microbiota and the role of commensal organisms in colonization resistance to CDI have been reviewed in detail elsewhere (Adamu and Lawley. 2013). Readily available mouse models of CDI include gnotobiotic/germ-free mice (Pawlowski *et al*. 2010; Reeves *et al*. 2012), SPF mice undergoing an antibiotic cocktail therapy to disrupt the normal gut flora (Chen, X. *et al*. 2008; Buonomo *et al*. 2016; Abt *et al*. 2015) and, most recently, single antibiotic pre-treatment to induce susceptibility to CDI (Buffie *et al*. 2012; Theriot *et al*. 2014).

2.5.2 Colon tumorigenesis in *Apc*$^{Min/+}$ mouse model. Human CRC development is classified as involving at least four crucial genetic changes targeting three tumor-suppressor genes *APC*, *SMAD4* and *TP53* and one oncogene *KRAS*; these genes are thought to dominate the evolutionary process of CRC histopathological sequences (Fodde *et al*. 2001). The *APC* gene is the primary checkpoint for the initiation of epithelial transformation that leads to the formation of aberrant crypt foci (ACF), the very earliest neoplasia (Powell 1992). The tumor suppressor protein encoded by the *APC* gene regulates the nuclear translocation of β-catenin, an important transcriptional co-activator in the Wnt signaling pathway (MacDonald *et al*. 2009). Earlier studies have shown that Wnt signaling is commonly activated in colon cancers (Deitrick and Pruitt 2016). The loss of wild-type APC functions, resulting typically from loss of or mutation of the *APC* genes on both alleles, is needed to activate Wnt/β-catenin signaling and promote the

transcription of target genes involved in cell proliferation, anti-apoptosis, and angiogenesis (Fodde *et al*. 2001; Deitrick and Pruitt 2016). *APC* gene mutations occur in more than 70% of the patients with colon adenomas, so that the *APC* gene is regarded as the most common gene responsible for CRC (Brenner *et al*. 2014). Heterozygous $Apc^{Min/+}$ mice develop multiple colon tumors at 8-12 weeks after ETBF colonization, but sham $Apc^{Min/+}$ mice display typically zero to few colon tumors. In the $Apc^{Min/+}$ mouse model used in our lab, called $Apc^{Min\Delta716/+}$, the *Apc* gene is heterozygous for a truncation at codon 716 of the *Apc* gene. While many *Apc* gene mutation models exist (Colnot *et al*. 2004; Hinoi *et al*. 2007), this model displays a modest increase in colon tumors at baseline compared to other $Apc^{Min/+}$ mouse models. The $Apc^{Min/+}$ mouse model is classically considered as a colon cancer model, although most tumors occur in the small intestine unless the mouse is colonized by a currently recognized pro-oncogenic bacterium such as ETBF. Thus, $Apc^{Min/+}$ mice at baseline poorly replicate human disease because intestinal polyposis is largely limited to the small intestine (Hinoi *et al*. 2007).

2.6 *C. difficile* and human colon cancer. The notoriety of *C. difficile* infection has escalated in recent years due to the epidemic of strain 027 worldwide, increased disease incidence and severity, and high CDI recurrence rates (Warny *et al*. 2005). As stated earlier, CDI commonly results in mild to severe colitis and may precipitate inflammatory bowel disease (Viswanathan *et al*. 2010; Kirby 2011; Abt *et al*. 2016). Although inflammation- or colitis-associated colon cancer has been extensively studied, a connection between *C. difficile*-induced colitis and colon cancer has not been proposed. Part of the reason may be that, to date, CDI has largely been appreciated to be an acute

colonic disease.

To date, we lack epidemiology studies designed to study the association between *C. difficile* colonization/infection and CRC development. Our literature search identified interesting findings described in two recent human CRC studies; namely, that a high occurrence of *Fusobacterium* (most are *F. nucleatum*) and *C. difficile* exists in CRC patients (Bullman *et al*. 2017; Fukugaiti *et al*. 2015). However, these two studies only analyzed 10-20 patients each and whether *C. difficile* colonization is identified in CRC patients requires using a bigger CRC database and additional human studies. It is also possible that *C. difficile* detection belongs to a subgroup of CRC patients not yet identified and in need of characterization. Previous studies indicate that cancer patients on chemotherapies, not limited to colorectal cancer, exhibit an increased risk of CDI thought to be due to their immunocompromised status, chemotherapy and/or antibiotic exposure compared with non-cancer patients (McDonald *et al*. 2018; Chopra *et al*. 2010). Some studies report that 19% of patients were colonized with toxigenic *C. difficile* on admission to oncology wards, and 16.1% of 205 preoperative CRC patients were positive for *C. difficile* predominantly (97%) toxigenic isolates (Zheng *et al*. 2017). Whether CRC patients who are immunocompromised are more susceptible to CDI or *C. difficile* colonization that contributes to the progression of human CRC remains to be determined. For initiation of CRC, it would likely be necessary for a patient to be colonized with *C. difficile* for many years; however, long-standing longitudinal studies of *C. difficile* colonization in humans are not available.

Initial molecular studies suggest that the mechanism(s) of the association between toxin B and tumorigenesis are controversial. On one hand, toxin B might promote tumorigenesis through EGFR signaling pathway as an earlier study found that toxin B activates the epidermal growth factor receptor (EGFR)-ERK-MAP signaling pathway in human colonic epithelial cells (Na *et al*. 2005), which has long been associated with a number of cancers including CRC. Notably, EGFR overexpression has been reported to occur in 60%-80% of human CRCs and is associated with a poor prognosis. On the other hand, recent studies suggest that toxin B competes with activators of Wnt protein receptors to block Wnt/β -catenin signaling activity (Tao *et al*. 2016; Chen, P. *et al*. 2018), indicating toxin B might have an antitumor effect. Whether *C. difficile* toxin B affects colon tumorigenesis *in vivo* needs additional study.

Chapter 3

C. difficile strain isolated from human CRC tumor mucosa enhances tumor formation in chronically colonized SPF $Apc^{Min\Delta 716/+}$ mice

3.1 Abstract

As a notorious gut infectious agent, *C. difficile* infection is the leading cause of antibiotic-associated diarrhea and has been a focus of nosocomial, antibiotic-associated acute colitis research. However, current studies have rarely connected *C. difficile* infection with chronic colitis and/or related chronic pathogenesis. *C. difficile* infection as a risk for colon cancer development is a new and novel research area. Previous work of the Sears laboratory demonstrated that human mucosal biofilm homogenates induce colon tumor formation after inoculation into GF $Apc^{Min\Delta 850/+}$ or $Apc^{Min\Delta 850/+}$;$Il10^{-/-}$ mice. In this study, we identified that *C. difficile* is present in some human colonic biofilms that enhanced tumorigenesis in GF $Apc^{Min\Delta 850/+}$ or $Apc^{Min\Delta 850/+}$;$Il10^{-/-}$ mice. Herein, we demonstrated that toxigenic *C. difficile* strains (particularly the CRC-associated isolate), but not a non-toxigenic strain, promote colon tumorigenesis in SPF $Apc^{Min\Delta 716/+}$ mice at 12 weeks after colonization. We further showed that *C. difficile* strains demonstrating persistent toxin production *in vivo* are more potent in enhancing tumor formation. Although small numbers of *C. difficile* were detected by FISH in the inner mucus layer of the colonized mouse colons, a densely packed bacterial biofilm is not assembled at 2 weeks after colonization in both GF wild-type mice and SPF $Apc^{Min\Delta 716/+}$ mice. Additionally, *C. difficile* spatial

localization in the colon is not restricted to the tumor microenvironment and, thus, chronic colonization with *C. difficile* and its associated chronic mucosal inflammation is proposed as a possible risk factor for colon tumorigenesis in humans.

3.2 Introduction

Despite advances in clinical medicine and diagnostic methods, *C. difficile* infection remains the leading cause of antibiotic-associated colitis and causes about half a million cases each year in the United States (Lessa *et al*. 2015). Risk factors for CDI include antibiotic exposure, advanced age, the use of gastric acid-suppressing drugs, hospitalization and host variables like immunodeficiency (Chopra *et al*. 2010; Rupnik *et al*. 2009). *C. difficile*-induced colitis became a surging public health problem due to increased disease severity associated with select *C. difficile* strains and several major CDI outbreaks over the past two decades (Warny *et al*. 2005; McDonald *et al*. 2018;). Compelling progress has been gained in studying CDI pathogenesis, however, current research mainly focuses on the acute onset of colitis or association with inflammatory bowel disease in both human populations (Marsh *et al*. 2012; Galdys *et al*. 2014) and animal models (Theriot *et al*. 2014; Buonomo and Petri 2016; Abt *et al*. 2015).

CDI recurrence is a challenging clinical problem as up to 35% of CDI patients experience a recurrence within 8 weeks of a previous episode (Marsh *et al*. 2012; Song and Kim 2019). As a spore-forming bacterium, *C. difficile* is highly resistant to common disinfectants (Sebaihia *et al*. 2006). Further, antibiotics such as metronidazole, fidaxomicin or vancomycin after treatment of at least 10 days may still fail to cure the

disease, in part, because these antibiotics are inactive against the *C. difficile* spore form. However, antibiotic treatment contributes to ongoing fecal 'dysbiosis' that renders patients at a higher risk of recurrent CDI due to relapse of infection with the same strain or reinfection by acquisition of a different *C. difficile* strain (Song and Kim 2019). It is hypothesized that recurrence may be, at least in part, due to the spore-forming ability and/or further persistence in a colon mucosal biofilm that may provide *C. difficile* with an environment in which resistance to antibiotics and avoidance of host immune responses occurs (Galdys *et al*. 2014). The ability of *C. difficile* to form a single species biofilm or polymicrobial biofilms *in vitro* (Soavelomandroso *et al*. 2017) has been reported since 2012 (Soavelomandroso *et al*. 2017; Dapa and Unnikrishnan 2013; Dapa *et al*. 2013), however very little is known about *C. difficile* biofilm formation *in vivo* and its impact on epithelial biology. Whether CDI recurrence is associated with or results in chronic colitis-associated diseases including colorectal cancer remains unclear.

CDI has been very rarely connected to colon cancer development although limited data suggest cancer patients tend to have a higher risk of *C. difficile* colonization or infection. A few earlier clinical studies suggest that about 20% cancer patients are positive for *C. difficile* predominantly with toxigenic isolates (Zheng *et al*. 2017; Chopra *et al*. 2010; Burgner *et al*. 1997). Whether CRC patients who are immunocompromised are more susceptible to CDI or whether *C. difficile* colonization precedes and contributes to the initiation and/or progression of human CRC remains unclear. Recently, Bullman's RNA-Seq analysis (Bullman *et al*. 2017) with paired CRC primary tumors and liver metastases identified *C. difficile* sequences in 8 of 10 CRC patients; most cases were also associated

with *Fusobacterium*. This study is restricted by the limited patient number enrolled and, further, did not validate the *C. difficile* detection such as with targeted qPCR or microbiology culture. A population-based epidemiology study is needed to inform the association between *C. difficile* and CRC, and to characterize the clinical and molecular features of the CRC subset in which *C. difficile* is detected including the prior historical context (such as having been diagnosed with CDI).

A previous study in our lab (Dejea *et al*. 2014) identified mucus-invasive bacterial biofilms in the human colonic mucosa including tumor mucosa, paired normal mucosa from the edge of the surgical resections and colonoscopy biopsies from healthy individuals. When inoculated into GF $Apc^{Min\Delta850/+}$ or $Apc^{Min\Delta850/+}$;$Il10^{-/-}$ mice and SPF $Apc^{Min\Delta716/+}$ mice, homogenates of each type of biofilm positive (BF+) mucosa enhanced colon carcinogenesis and colonic biofilm assembly as compared with biofilm negative (BF-) biopsies (Tomkovich *et al*. 2019). Using 16S rRNA amplicon sequencing, we identified *C. difficile* in BF+ human inocula as well as stools and colons from mice inoculated with the human BF+ inocula in which *C. difficile* was detected, but not in mice inoculated with BF- mucosa homogenate (Fig. 1A). These results led us to ask whether *C. difficile* is a driver species in the biofilm microbiota that is carcinogenic and bacterial biofilm-promoting in $Apc^{Min/+}$ mouse models. Herein, this study will shed light on the mechanisms and role of *C. difficile* in BF+ human CRC development.

In this project, we hypothesized that *C. difficile* colonization promotes a pro-carcinogenic environment in the colons of $Apc^{Min/+}$ mice. To test this hypothesis, we developed a new

sustainable and non-lethal mouse model for *C. difficile* chronic colonization using SPF $Apc^{Min\Delta716/+}$ mice. We further tested the capability of *C. difficile* to assemble single-species biofilms in GF wild-type C57BL/6J mouse colons or polymicrobial species biofilms in SPF $Apc^{Min\Delta716/+}$ C57BL/6J mouse colons.

3.3 Material and Methods

3.3.1 DNA extraction and 16S rRNA amplicon sequencing. These methods have been described in a previous publication (Tomkovich *et al*. 2019). In brief, DNA was extracted from human mucosal homogenates, mouse feces (collected at week 1 and week 12) and distal colon tissue snips (week 12) from colonized GF $Apc^{Min\Delta850/+}$ and $Apc^{Min\Delta850/+}$;$Il10^{-/-}$ mice using phenol/chloroform separation followed by DNeasy Blood & Tissue Kit (QIAGEN). To construct DNA libraries for 16S rRNA amplicon sequencing, we targeted the V1–V3 hypervariable region of 16S rRNA gene for PCR amplification using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 534R (5′-ATTACCGCGGCTGCTG G-3′). The primer pairs are labeled with universal Illumina paired-end adapter sequences and unique barcodes of 4 to 6 nucleotides to identify individual targets in multiplex sequencing. PCR amplicons were purified using the Agencourt AMPure XP Kit (Beckman Coulter Inc.) and quantified with the KAPA Library Quantification Kit (KAPA Biosystems) (Tomkovich *et al*. 2019). Samples were pooled in equimolar amounts and sequenced on an Illumina MiSeq according to the manufacturer's specifications. 16S rRNA sequences are available in the NCBI's Sequence Read Archive (SRA PRJNA423288).16S rRNA-Seq data were analyzed by Dr. James White using the Resphera Insight high-resolution protocol (Drewes *et al*. 2017; Tomkovich *et al*. 2019). Samples with more than 14,000 high-quality

sequences were randomly subsampled to 14,000 per sample. Relative abundance estimates are based on the subsampled dataset. High-quality passing sequences were assigned to a taxonomic lineage using the Resphera Insight method that has been optimized to ascribe corresponding sequences to species level membership. Single species assignments are designed to be of diagnostic confidence in quality.

3.3.2 *C. difficile* human strain isolation. As stated in 3.3.1, human biofilm microbiota were transferred to GF $Apc^{Min\Delta 850/+}$ or $Apc^{Min\Delta 850/+}$;$Il10^{-/-}$ mice and the microbial composition in mouse feces was profiled using 16S rRNA amplicon sequencing. Based on its comparatively high *C. difficile* relative abundance, we picked week 1 stool from mouse #2663 for further analysis. The frozen mouse stool (-80℃) was first homogenized anaerobically in CCMB-TAL medium (Cycloserine Cefoxitin Mannitol Broth with Taurocholate Lysozyme Cysteine, Anaerobe Systems) that is predicted to help *C. difficile* spores recover and support germination. After 48 hours, turbidity and a visible color change of CCMB-TAL medium from pink to yellow indicated spore germination and *C. difficile* growth. Next, the suggestive CCMB-TAL broth was streaked on CCFA-HT agar plates (Cycloserine Cefoxitin Fructose Agar with Horse Blood and Taurocholate, Anaerobe Systems). Single colonies grown on CCFA-HT plates were genotyped by colony qPCR performed on QuantStudio 5 Real-Time PCR Systems (Thermo Fisher) with primers targeting the *C. difficile* species-specific 16S rRNA gene region as well as *tcdA* and *tcdB* genes (Table 1). The *C. difficile* 16S rRNA gene was amplified in a qPCR reaction mixture of 0.4 μM primers, 0.2 μM fluorescent probe (Table 1), 1x TaqMan™

Table 1. *C. difficile* primers and probes used in this study		
C. difficile 16s rRNA-forward	TTGAGCGATTTACTTCGGTAAAGA	(Rinttilä *et al.* 2004)
C. difficile 16s rRNA-reverse	CCATCCTGTACTGGCTCACCT	
C. difficile probe 16S_probe_TAMRA_IBRQ	TAACGCGTGGGTAACCTACCCTGTA	Designed for this study using IDT tool
C. difficile tcdA-forward-qPCR	GTCGGATTGCAAGTAATTGACAATA	(Lyras *et al.* 2009)
C. difficile tcdA-reverse-qPCR	TAACAGTCTGCCAACCTTTTGAGA	
C. difficile tcdB-forward-qPCR	ACCATATAGCTTTGTAGATAGTGAAGGAAA	
C. difficile tcdB-reverse-qPCR	AAGAACTACATCAGGTAATTCAGATACAAA	

Gene Expression Master Mix (Thermo Fisher), and bacterial DNA using the following conditions: 95℃ for 10 min and 40 cycles of 95℃ for 30s and 58℃ for 60s. *tcdA* and *tcdB* genes were detected using a qPCR reaction of 0.4 μM primers, 1x Fast SYBR® Green Master Mix, and bacterial DNA under the conditions: 95℃ for 20 min and 40 cycles of 95℃ for 3s and 58℃ for 30 s.

3.3.3 *C. difficile* strains and spore preparation. *C. difficile* strains used in this study are summarized in Table 3 and the toxin genes were typed by qPCR using the primers in Table 1. The toxigenic strain ATCC 9689 and non-toxigenic strain ATCC 700057 were kindly provided by the Clinical Microbiology Laboratory of The Johns Hopkins Hospital. The ribotyping of CRC-associated isolate CI_m_2663 was performed by Dr. Seth Walk at Montana state University by fluorescent PCR ribotyping as previously detailed (Walk *et al*. 2012). The whole genomes of *C. difficile* strains including ATCC 700057, and CI_m_2663 (human strain passed through GF mouse gut) were sequenced by The CHOP Microbiome Program at University of Pennsylvania and analyzed by Dr. James White in alignment with whole genome sequences of a few reference strains available in NCBI's database including strain ATCC 9689 which is also included in this study.

For spore preparation, *C. difficile* strains were plated to a lawn on reduced BHI (Brain Heart Infusion Powder, Becton Dickinson) plates and cultured anaerobically for 5 to 8 days at 37°C to induce sporulation. Colonies were collected in 1-2 ml sterile phosphate-buffered saline (PBS) and incubated at 60°C for 1 hour to kill the vegetative cells. Spores were washed 3 times with PBS, and viable spores were enumerated as colony-forming units (CFUs)/ml by plating serial dilutions on CCFA-HT plates or BHI-T (BHI agar supplemented with 0.1% taurocholate (Sigma)) plates.

3.3.4 Mouse breeding and housing. The male *Apc*$^{Min\Delta716/+}$ C57BL/6J mice (6-week- to 6-month-old, in-house breeding colony) and female wild-type C57BL/6J mice (6-week- to 9-month-old, Stock No. 000664, Jackson laboratories) were housed and bred with autoclaved food, bedding and chlorinated water in the specific-pathogen-free (SPF) facility in the Cancer Research Building II at Johns Hopkins University. Germ-free **(**GF) C57BL/6J wild-type mice were housed and bred in flexible film isolators with sterile water, diet, bedding and filtered air in the Germfree Mouse Core Facility (under SOP SP20M233 protocol) at Johns Hopkins University. All animal procedures were performed in accordance with the NIH guidelines for housing and care of laboratory animals and were approved by the Johns Hopkins University Animal Care and Exposure Committee (under protocol MO20M85).

3.3.5 Mouse experiment SPF *Apc*$^{Min\Delta716/+}$ mice, 6- to 10-week-old, both male and female, were given vancomycin (Sigma) (0.05 mg/ml) and gentamicin (Sigma) (0.035 mg/ml) in drinking water for 5-7 days (range from 3 to 5 days in pilot studies) followed by 2-3 days

of regular drinking water prior to the inoculation of 10^6 *C. difficile* spores (range from 10^3 to 10^6 in pilot studies) in PBS or PBS alone for sham mice via oral gavage. Gentamicin (0.035 mg/ml) supplement in the drinking water was resumed after inoculation and continued throughout 3 months of the experiment duration. Germ-free wild-type mice, 10- to 12-week-old, both male and female, were inoculated with 10^4 *C. difficile* spores in PBS. We monitored signs of illness or disease including survival, body weight loss, diarrhea, bloody stools and hunched posture. To avoid cross-contamination, sham germ-free wild-type mice were kept in isolators until necropsy.

The level of *C. difficile* colonization in experimental mice was evaluated from fecal samples as follows. In the first few pilot experiments, we extracted DNA from week 4 and/or week 12 stools using Quick-DNA Fecal/Soil Microbe DNA Miniprep Kit (Zymo Research) and quantified *C. difficile* copy numbers by qPCR with *C. difficile* 16S rRNA primers and probe as described above. In subsequent experiments, longitudinal *C. difficile* bacterial burden was determined by both qPCR (at week 1, week 4 and week 12 after colonization) and microbial culture (at week 1, week 4, week 8 and week12 after colonization). To cultivate *C. difficile* from mouse stools, ~ 0.02-0.10 g stool samples (1 g/ml, e.g., 0.1 g stool = 0.1 ml) were suspended in sterile and preconditioned PBS (9 parts of stool volume, e.g., 0.9 ml for 0.1 g stool) in an anaerobic chamber. Stool homogenates were heated at 60°C for 1-2 hour to remove vegetative cells and serially diluted for *C. difficile* enumeration (CFUs/g stool) on CCFA-HT or BHIS-T (BHI agar supplemented with *Clostridium difficile* Supplement (Sigma) and 0.1% taurocholate) plates.

3.3.6 Colon harvest and tumor assessment. Mice were necropsied at 12 or 13 weeks after inoculation. Colons were flushed with sterile PBS and cut open longitudinally to grossly examine colonic tumors. For mucosal cytokine analysis, a piece of middle colon (about 0.5 cm), free of visible tumors, was flash-frozen in liquid nitrogen. The remaining colon was fixed in 10% neutral buffered formalin solution overnight followed by methylene blue staining. Colonic tumors were hereby visualized as dark blue and quantified under the Leica ES2 Stereo Microscope by one or two experienced experts separately (S.W. or C.L.S.). For histopathology, the colons were Swiss-rolled for paraffin-embedding, sectioning (4 μm) and Hematoxylin and Eosin (H&E) staining by Oncology Tissue Services (OTS) Core Facility at the Johns Hopkins University. Histological scoring of inflammation, epithelial damage, hyper-proliferation and microadenoma quantification was performed blindly by S.W.

3.3.7 Tissue RNA extraction, cDNA synthesis, and RT-qPCR. Total RNA from 0.5 cm normal middle colon tissue was extracted using the Direct-Zol™ RNA Miniprep kit (Zymo Research) following the protocol. In brief, flash-frozen tissue snips were thawed to room temperature in RNase and DNase free tubes containing TRIzol® and 2 mm Zirconia-Silicate beads and then homogenized by bead beating at 3800 rpm for 2 min. Tissue homogenates were centrifuged at 4°C, 10,000 g for 5 min to remove tissue debris. Total RNA was purified from the clarified homogenates by passing through a Zymo-Spin Column and RNA binding column sequentially with wash and elution buffers, and the RNA concentration was measured by a NanoDrop Spectrophotometer (Thermo Scientific). Then, we used 500ng or 1μg of RNA for cDNA reverse transcription using the High-

Capacity RNA-to-cDNA Kit (Thermo Scientific). The target genes evaluated by the TaqMan™ Gene Expression Assay (FAM) are listed in Table 5. The relative mRNA quantities were expressed using a standard ΔΔCT method that calculated fold-changes and were normalized to mouse GAPDH, a housekeeping gene.

3.3.8 Fluorescence *in situ* hybridization (FISH) and confocal microscopy. The distal 3 cm of colons with fecal content from SPF $Apc^{Min\Delta716/+}$ and GF wild-type mice were immediately fixed in Carnoy's solution (60% methanol, 30% acetic acid, and 10% chloroform) and followed by paraffin-embedding, and sectioning (4 μm) in the OTS core facility as described above. Bacterial spatial localization and/or biofilm formation was visualized on colon sections by FISH with the EUB338 universal bacterial probe (5'-GCTGCCTCCCGTAGGAGT-3') and *C. difficile* species-specific probe (5'-CATCCTGTACTGGCTCAC-3') contrasted with nuclear counterstain, 4′,6-diamidino-2-phenylindole (DAPI). In brief, unstained colon slides were deparaffinized through baking at 50°C for 10 min and 2 dips in xylene followed by re-hydration through an ethanol serial gradient. 0.5 ng/μl pre-mixed bacterial probes in hybridization buffer containing 20 mM Tris-HCl, 0.9 M NaCl, 0.01% sodium dodecyl sulfate were applied to the slides and then incubated in a dark humidified chamber at 46°C for 1.5 hours. The hybridized slides were covered with ProLong Gold® Antifade Mountant (Cell Signaling Technology) and stored in the dark at room temperature prior to confocal microscopy. Confocal microscopy (Zeiss LSM880) was conducted in the Microscope Core Facility at Johns Hopkins School of Medicine, and images were analyzed by ImageJ (NIH). Bacterial biofilms on Carnoy's fixed mouse colonic tissues were defined as a dense bacterial community invading the

inner mucus layer (within 1 μm of the epithelium) for at least 200 μm of the epithelial surface (Dejea *et al*. 2014; Tomkovich *et al*. 2019).

3.3.9 Periodic Acid-Schiff (PAS) stain. Following the protocol of the PAS Stain Kit (Sigma-Aldrich), we stained the distal 3 cm colon sections to assess mucus presence in parallel with successive sections hybridized with FISH bacterial probes. In brief, colon slides were deparaffinized and re-hydrated as stated in the FISH procedure (Material and Method 3.3.8). Then, the slides were sequentially immersed in 1% Periodic Acid Solution for 10 min and Schiff's Reagent for 40 min with rinse steps in distilled water after each stain. Hematoxylin Solution (Mayer's, Modified) was used for nuclear counterstaining.

3.3.10 Statistical Analysis. Statistical analysis was conducted with GraphPad Prism software version 7.0. Numerical data without a normal distribution were expressed as means ± SE, and differences between two groups were evaluated using the nonparametric Mann-Whitney U test. For multiple group comparisons, statistical analysis was performed with nonparametric Kruskai-Wallis test followed by the uncorrected Dunn's test for each two-group comparison because we considered each experimental group as independent from others. Differences of $P < 0.05$ were considered statistically significant.

3.4 Results

3.4.1 Detection, isolation and characterization of a *C. difficile* strain from human biofilm-positive tissue and subsequently inoculated mice. Our previous translational

study (Tomkovich *et al*. 2019) that transferred human mucosal homogenates to GF *Apc*$^{Min\Delta850/+}$ or *Apc*$^{Min\Delta850/+}$;*Il10*$^{-/-}$ mice was conducted in collaboration with the laboratory of Dr. Christian Jobin at the University of Florida. In brief, GF *Apc*$^{Min\Delta850/+}$ or *Apc*$^{Min\Delta850/+}$;*Il10*$^{-/-}$ mice were inoculated with 4 types of human colonic mucosal homogenates: biofilm-positive tumors (BF+T) and biofilm-positive paired normal tissues (BF+NF) from the surgical resections of 5 CRC patients or histologically normal biofilm-positive biopsies (BF+bx) or biofilm-negative (BF-bx) biopsies from 5 healthy individuals who underwent screening colonoscopy. After inoculation, mouse fecal pellets were collected at week 1 and week 12. Colon snips were flash-frozen at week 12 during the mouse harvest. The microbial composition in the human inocula and the subsequently inoculated mouse feces (at week 1 and week 12) and flash-frozen mouse colon mucosa (at week 12) were profiled using 16s rRNA amplicon sequencing as detailed in Materials and Methods 3.3.1. Unexpectedly, we identified *C. difficile* sequences in mice inoculated with biofilm-positive human mucosal homogenates, but not in mice inoculated with a biofilm-negative human mucosal homogenate (Figure 3.1). The *C. difficile* relative abundance was selectively enriched in mouse stools and colonic tissues as compared to the human colonic mucosal inocula that were pooled from 5 individual patients respectively in each of the groups, BF+bx, BF+NF, BF+T as well as BF-bx. Further, 16S rRNA amplicon sequencing analysis of individual patient colonic mucosa identified that one of the 5 biofilm-positive tumors was *C. difficile* positive (19/14000 reads in patient 3728-T3, Table 2). This result was validated using *C. difficile* selective culture followed by colony PCR for the *tcdB* gene. In contrast, we did not identify a *C. difficile*-positive individual patient in BF+NF group based on either 16S rRNA amplicon sequencing results

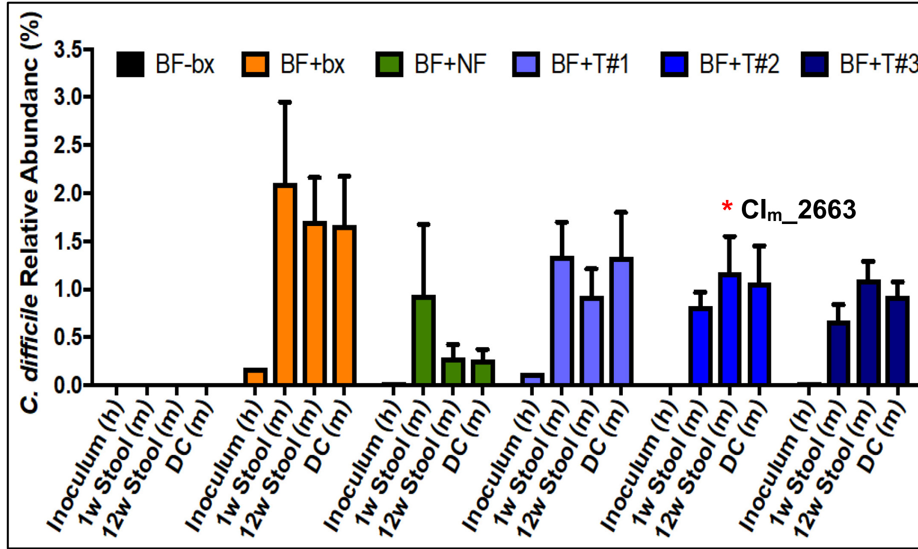


Figure 3.1 *C. difficile* is detected persistently in human biofilm-positive mucosa and subsequently colonized germ-free mice that develop colon tumors. 16S rRNA amplicon sequencing was done with DNA extracted from human (h) mucosal inoculum, mouse (m) stools (at 1 week and 12 weeks) and mouse colonic tissues (at 12 weeks). Human mucosae included biofilm-negative biopsies (BF-bx), biofilm-positive biopsies (BF+ bx), biofilm-positive normal flanking (BF+ NF), and biofilm-positive tumors (BF+ T#1, BF+ T#2, BF+ T#3). Each type of human mucosal homogenate inoculum was a mixture combined from 5 patients of each group. T#1, T#2 and T#3 were three replicates of tumor homogenates made from the same 5 patients. Relative abundance of Operational Taxonomic Units (OTUs) (Y axis) from 16S rRNA amplicon sequencing was calculated as the percentage of absolute reads out of 14000 randomly normalized total reads. Cl_m_2663 (the red star) is the human CRC-associated *C. difficile* isolate cultured from one of the mice (stool at 12 weeks) inoculated with the BF+ T#2 human mucosal homogenate. The relative abundance of *C. difficile* in each type of human inoculum was 0.186% (BF+ bx), 0.007% (BF+ NF), 0.129% (BF+ T#1), 0 (BF+ T#2) and 0.007% (BF+

Table 2. *C. difficile* validation with individual human mucosa from tumors or normal flanking regions

	BF+ Tumor inoculum (#1, 2, 3) *				
	3728-T3	3753-T	3754-T	3756-T	3774-T
***C. difficile* 16S rRNA Seq**	19/14000	0	0	0	0
C. difficile* culture** **Colony PCR for *tcdB	+ +	Not detected	Not detected	Not detected	Not detected
BF+ Tumor inoculum (#1, 2, 3) * were 3 replicates of human tumor mucosa homogenates combined from the same 5 patients.					
	BF+ NF inoculum				
	3726-NF	3753-NF	3754-NF	3756-NF	3774-NF
***C. difficile* 16S rRNA Seq**	0	0	0	0	0
C. difficile* culture** **Colony PCR for *tcdB	Not detected	Not detected	Not detected	Not detected	Not detected

or microbiology culture.

The mouse stool specimens (provided by our collaborator, Dr. Sarah Tomkovich in the laboratory of Dr. Christian Jobin), in which *C. difficile* was detected by sequencing, were used to recover the *C. difficile* strain. We successfully isolated a *C. difficile* strain from the 12-week stool of GF mouse #2663 that was inoculated with human BF+T mucosa and named this strain as CRC isolate CI_m_2663. This isolate was confirmed as a toxigenic *C. difficile* strain by colony qPCR using the primers/probe for *C. difficile* 16S rRNA gene (TagMan) and primers for *tcdA* and *tcdB* (SYBR green). To compare with additional *C. difficile* strains in further experiments, we obtained two ATCC strains, the toxigenic *C. difficile* strain ATCC 9686 and non-toxigenic strain ATCC 700057 (Table 3) and confirmed *C. difficile* 16S rRNA, *tcdA* and *tcdB* genes in these strains using qPCR.

Table 3. Toxin genes and ribotypes of *C. difficile* strains used in this study

Strains	***tcdA***	***tcdB***	**Ribotype**	**Source**
Non-toxigenic ATCC 700057	-	-	038	Human
Toxigenic ATCC 9689	+	+	001	Human
Toxigenic CI_m_2663 (Human CRC-associated isolate)	+	+	FP485	Human CRC-associated strain passed through germ-free mice
Toxigenic 630Δ*erm* wild type	+	+	012 (Refer to strain 630)	Derivative of human strain 630
***tcdB*$^-$ 630Δ*erm* mutant**	+	-	012 (Refer to strain 630)	Mutant on 630Δ*erm* background

3.4.2 Establish chronic colonization of *C. difficile* in SPF *Apc*$^{Min\Delta716/+}$ mouse model.

To test the colonic tumorigenesis potential of our CRC isolate CI_m_2663 in mice, we needed to establish a murine model displaying *C. difficile* chronic colonization for 3

months. This model differs from the current CDI murine models that exhibit robust acute colitis with up to 40%-50% mortality within the first few days after infection (Chen, X. *et al*. 2008; Buonomo *et al*. 2016; Abt *et al*. 2015). In the first few pilot studies using 6-8-week old SPF *Apc*$^{Min\Delta716/+}$ mice (all *Apc*$^{Min/+}$ hereafter will be *Apc*$^{Min\Delta716/+}$) in both genders, we tested an antibiotic cocktail (Buonomo *et al*. 2016; Abt *et al*. 2015) modified from reports on current CDI murine models, and initiated a new treatment regime of vancomycin (0.050 g/L) and gentamicin (0.035 g/L) in drinking water for 3 days, followed by orally inoculation with 10^3 -10^6 CFUs of *C. difficile* spores and gentamicin (0.035 g/L) in the drinking water for 12 weeks . The results of fecal culture and species-specific 16S rRNA PCR for *C. difficile* showed that the colonization of *C. difficile* strains (Table 3) was not consistently sustained for 12 weeks (Figure 3.2 A).

The 12-week endpoint colonization ratio was 10% (1/10 mice) for strain ATCC 700057, 50% (9/18 mice) for strain ATCC 9689 and 27% (3/11 mice) for CRC isolate CI_m_2663 (Figure 3.2B). Subsequently, we improved the colonization persistence by refreshing the vancomycin and gentamicin supplemented water every 24-36 hour to maintain antibiotic effectiveness for 5-7 days before inoculation while using the same antibiotic formula and the number of *C. difficile* spores for inoculation. Utilizing microbial culture (Figure 3.2C) and qPCR (Figure 3.2D), we demonstrated that fecal colonization levels of the three *C. difficile* strains were similar once established and persisted across multiple time points during the 12-week study course. The colonization ratio was increased to 50% (4/8 mice) for strain ATCC 700057 up to 12 weeks, 100% (8/8 mice) for strain ATCC 9689 up to 12 weeks, and 100% (8/8 mice) for strain CRC CI_m_2663 until 8 weeks, then declined to 63%

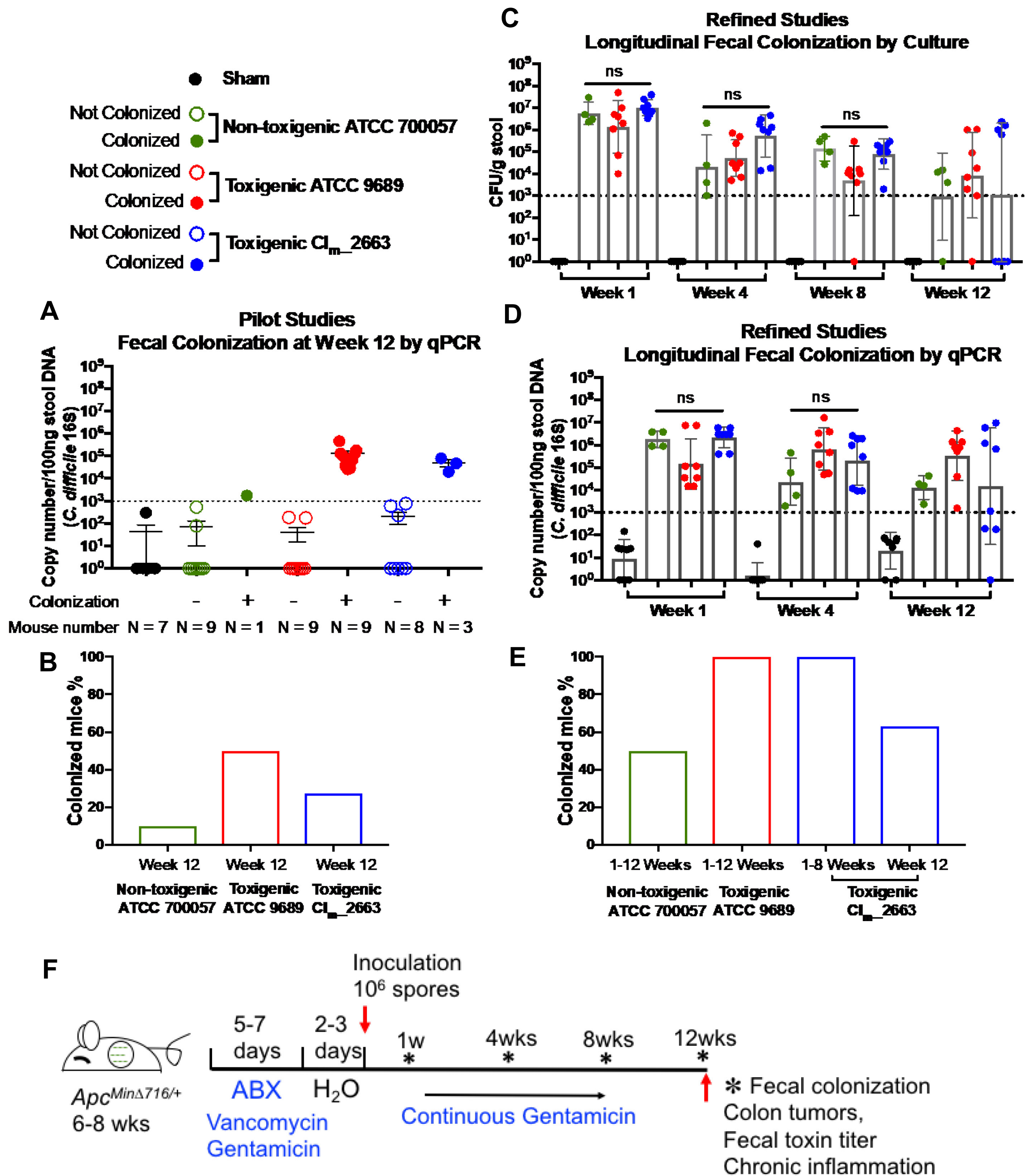


Figure 3.2 New antibiotic pretreatment facilitates *C. difficile* chronic colonization in SPF *Apc*$^{Min\Delta716/+}$ mice. A-B. Fecal colonization was detected using qPCR (A) and the ratio of colonized mice at 12 weeks in the first few pilot studies. C-E. Fecal colonization was determined by microbial culture (C) and qPCR (D) at multiple time points during 12 weeks in two refined experiments. 10^3 was the detection limit of both microbial culture and qPCR. Any value below 10^3 by qPCR (D) is considered *C. difficile* negative, and this is validated by culture (C). E. The ratio of colonized mice in the two refined experiments. F. Schematic diagram of establishing chronic colonization of *C. difficile* in SPF *Apc*$^{Min\Delta716/+}$ mice.

(5/8 mice) by Week 12 (Figure 2.3E). Unexpectedly, in contrast to the successful colonization of *C. difficile* strains in SPF *Apc*$^{Min\Delta716/+}$ mice, SPF wild-type mice under the same protocol of antibiotic treatment displayed colonization resistance or lost the initial colonization after 1 week. Thus, we were unable to establish a chronic colonization model of *C. difficile* in SPF wild-type mice.

3.4.3 Toxigenic *C. difficile* strains enhance colon tumor formation in SPF *Apc*$^{Min\Delta716/+}$ mice. To test whether *C. difficile* modified tumorigenesis, we inoculated *Apc*$^{Min\Delta716/+}$ mice with the CRC isolate CI_m_2663 and in parallel also tested the non-toxigenic strain ATCC 700057 and toxigenic strain ATCC 9689. We monitored the mouse body weight and colitis symptoms like diarrhea or bloody stool. At 12 weeks, colon tumors were examined after methylene blue staining as described in Materials and Methods 3.3.6. As shown in Figure 3.3A and B, the toxigenic *C. difficile* strains of CRC isolate CI_m_2663 and ATCC 9689 together significantly enhanced colon tumor formation as compared with the sham mice (P = 0.0119) and non-toxigenic strain ATCC 700057 (P = 0.0096) with similar tumor sizes (data not shown). When analyzed separately, our human CRC isolate CI_m_2663 promoted more tumors than sham (P = 0.0198) and non-toxigenic strain ATCC 700057 (P = 0.0126) (Figure 3.3C). The toxigenic *C. difficile* strain ATCC 9689 also increased tumor incidence in a subgroup of mice, although the statistical difference was only significant when compared with non-toxigenic strain ATCC 700057 (P = 0.0419). Of note, both toxigenic *C. difficile* strains predominantly increased tumor formation in the distal colon of SPF *Apc*$^{Min\Delta716/+}$ mice. Microadenoma counts examined on H&E-stained mouse colon sections showed no difference across the groups at 12 weeks (Figure 3.3D).

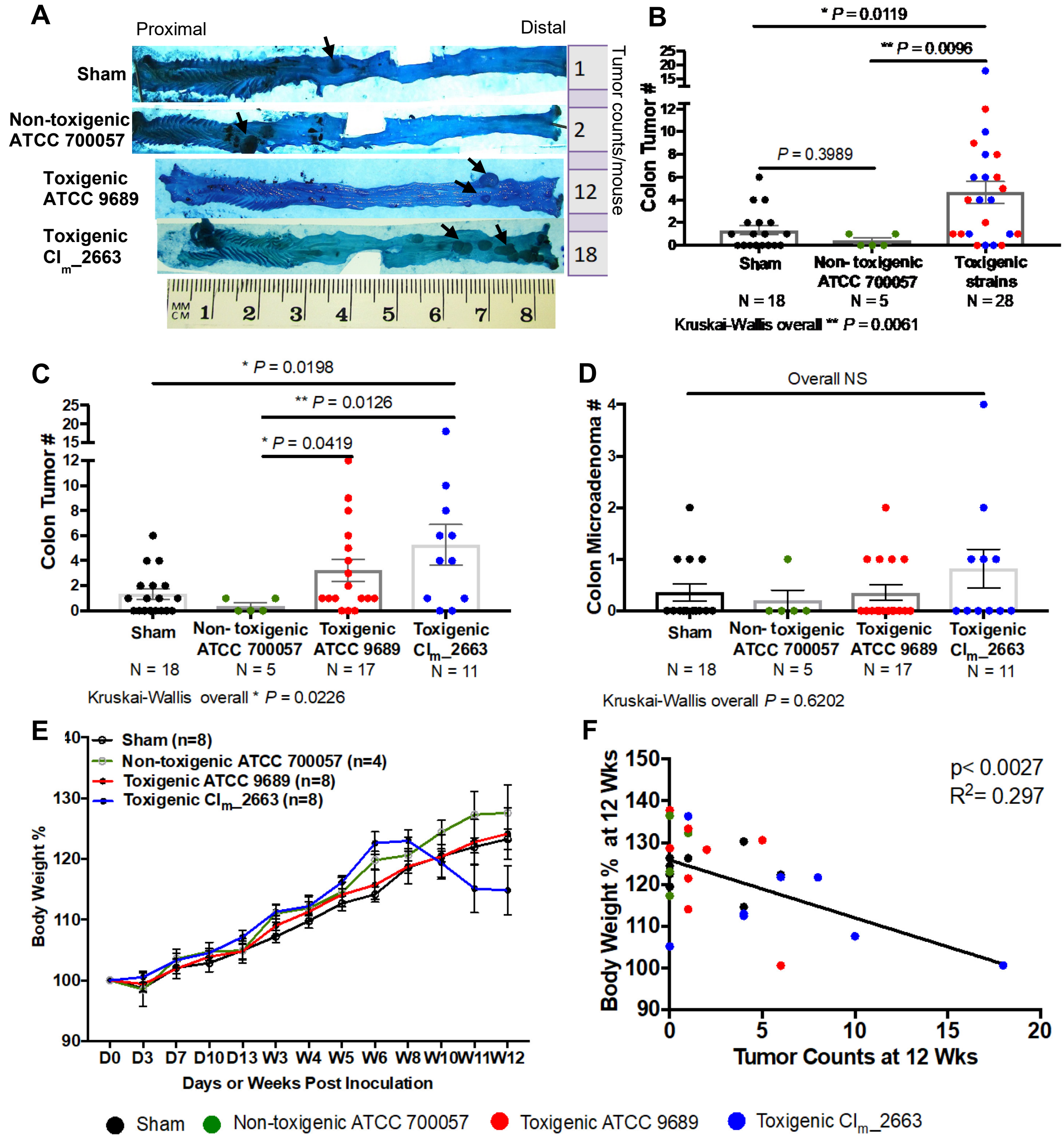
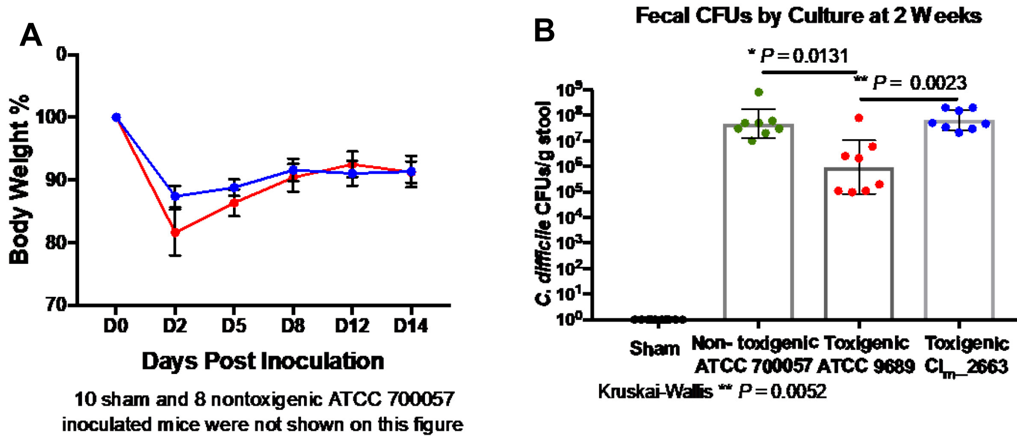


Figure 3.3 Toxigenic *C. difficile* strains enhance colon tumor formation in *Apc*$^{Min\Delta716/+}$ mice at 12 weeks after inoculation. A. Representative pictures of mouse colons that were stained with methylene blue; B-C. Mouse colon tumor numbers were shown with toxigenic strains combined (B) or separated (C); D. Microadenoma counts by microscopy on H&E-stained mouse colon sections; E. No acute colitis signs including significant early body weight loss were observed within the first week after *C. difficile* inoculation in SPF *Apc*$^{Min\Delta716/+}$ mice; F. Later body weight loss, particularly in mice inoculated with toxigenic clinical isolate Cl_m_2663 strain after 8 weeks was shown to correlate with tumor burden. The correlation was performed by linear regression analysis. Data represent at least 3 independent mouse experiments for each group. The overall *P* value among multiple groups was calculated using Kruskai-Wallis test followed by uncorrected Dunn's test for the comparison of two group. A *P* value < 0.05 is considered significant.

Overall, the colonization of *C. difficile* strains under our adjusted antibiotic treatment did not lead to any acute death, or rapid onset of symptomatic diarrhea with body weight loss (Figure 3.3E). The body weight loss in later time course correlated with tumor counts in the mouse colons (Figure 3.3F).

3.4.4 *C. difficile* colonized SPF *Apc*$^{Min\Delta716/+}$ mice show different morbidity and inflammatory features as compared with an acute colitis model using GF wild-type mice analyzed at two weeks after inoculation. To better characterize our mouse model of *C. difficile* chronic colonization, we evaluated the disease at the acute phase in SPF *Apc*$^{Min\Delta716/+}$ mice and GF wild-type mice. The mouse body weights were monitored frequently for the first 2 weeks post-inoculation showed that GF mice inoculated with toxigenic *C. difficile* strains ATCC 9689 or CRC isolate CI_m_2663 demonstrated about 20% body weight loss (Figure 3.4A), diarrhea, hunched posture and agitation during the first 2-5 days after the inoculation. Sham GF mice and non-toxigenic *C. difficile* strain inoculated mice did not show any morbidity consistent with acute colitis. The body weights for GF sham mice were not monitored to avoid cross contamination from repeatedly opening the cages under germ-free condition. The average fecal colonization levels after 2 weeks in *C. difficile* inoculated GF mice (Figure 3.4B) were in the range of 10^6 to 10^8 CFUs/g stool. Toxigenic strain ATCC 9689 showed less *C. difficile* burden in feces relative to strain ATCC 700057 and CRC isolate CI_m_2663 but displayed more body weight loss (Figure 3.4A-B). This suggested that fecal bacterial load alone did not fully explain the variations in disease severity. Moreover, we did not observe significant body weight loss in SPF *Apc*$^{Min\Delta716/+}$ mice during the initial 2 weeks of *C. difficile* colonization (Figure 3.4C-

GF wild-type mice



SPF $Apc^{Min\Delta716/+}$ mice

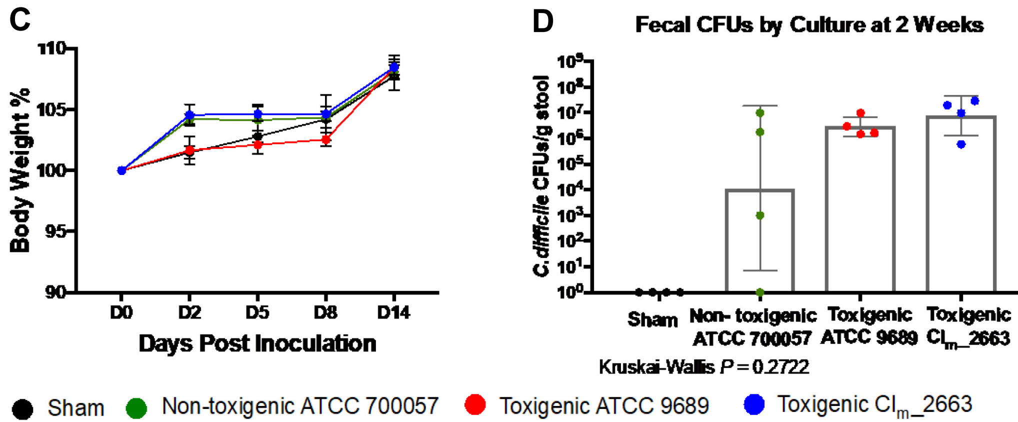
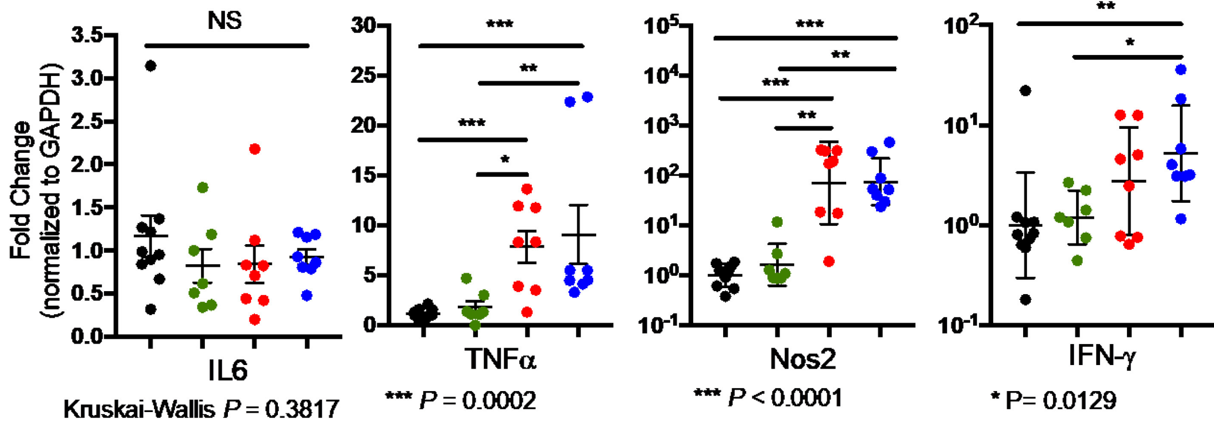


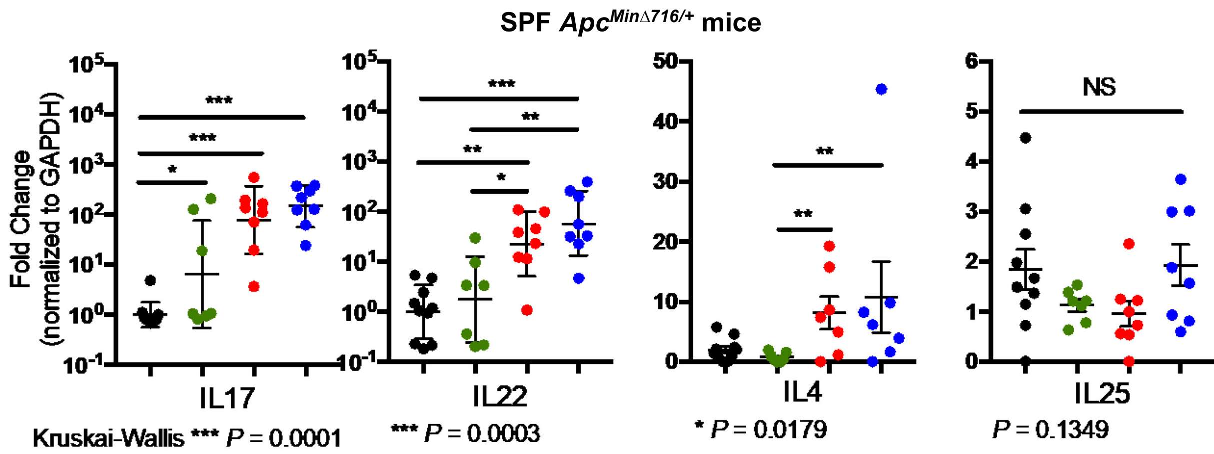
Figure 3.4 Morbidity and bacterial burden during/at 2 weeks after *C. difficile* inoculation in GF wild-type mice and SPF $Apc^{Min\Delta716/+}$ mice. GF wild-type mice were challenged with 10^4 spores and SPF $Apc^{Min\Delta716/+}$ mice were challenged with 10^6 spores. Body weight is presented as percent of the original body weight. A-B: Body weight % (A) and fecal colonization (B) in GF wild-type mice inoculated with toxigenic *C. difficile* strains ATCC9689 (n=8) and CRC CI_m_2663 (n=8). The body weights of GF sham mice (n=10) and mice inoculated with non-toxigenic ATCC700057 strain (n=8) were not documented due to lack of disease symptoms, and to maintain GF status of sham mice or avoid contamination for mice inoculated with non-toxigenic strain. C-D: Body weight % (C) and fecal colonization (D) in SPF $Apc^{Min\Delta716/+}$ mice. GF data represent two independent experiments, and SPF data represent one single experiment.

D). Similar to the colonization outcomes in the 12-week colon tumor experiment (Figure 3.2), non-toxigenic strain ATCC70057 colonized modestly less well in SPF *Apc*$^{Min\Delta716/+}$ mice, while the two toxigenic *C. difficile* strains showed similar level of bacterial loads at 10^6-10^7 CFUs/g stool at two weeks (Figure 3.4D) when compared with GF mice colonized for two weeks with *C. difficile* (Figure 3.4B). We predicted that the differing morbid manifestations between GF wild-type and SPF *Apc*$^{Min\Delta716/+}$ mice during the first 2 weeks after infection reflected differential inflammatory responses, suggesting that the host mucosal environment impacts disease severity in response to *C. difficile* infection. In addition, we hypothesized that the sub-acute inflammatory characteristics at 2 weeks after *C. difficile* inoculation may persist in the long term and lead to a pro-tumorigenic environment in *Apc*$^{Min\Delta716/+}$ mice.

We aimed to profile and compare the colonic tissue-derived cytokine expression by qPCR with 0.5 cm middle colon tissues from these two mouse models. As noted, GF wild-type mice appeared ill within the first week after infection. Although symptoms appeared resolved at 2 weeks, qPCR assay of colonic tissue demonstrated significant upregulation of TNF-α, Nos2, IFN-γ, IL-17A, IL-22 and IL-4 upon colonization with toxigenic *C. difficile*, particularly CRC isolate CI_m_2663 (Figure 3.5). Increased IL-17A expression was mainly driven by toxigenic *C. difficile* although the non-toxigenic strain also stimulated IL-17A expression in some mice. We did not observe a downregulation of IL-25 as previously shown by other studies using human colonic tissues from CDI patients and antibiotic-pretreated SPF mouse models (Buonomo *et al*. 2016). In contrast, when assayed using the same qPCR panel, colonic cytokine expression in SPF *Apc*$^{Min\Delta716/+}$ mice at 2 weeks

A GF wild-type mice





● Sham ● Non-toxigenic ATCC 700057 ● Toxigenic ATCC 9689 ● Toxigenic Cl_m_2663

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Figure 3.5 Continued on Next Page

B SPF $Apc^{Min\Delta716/+}$ mice

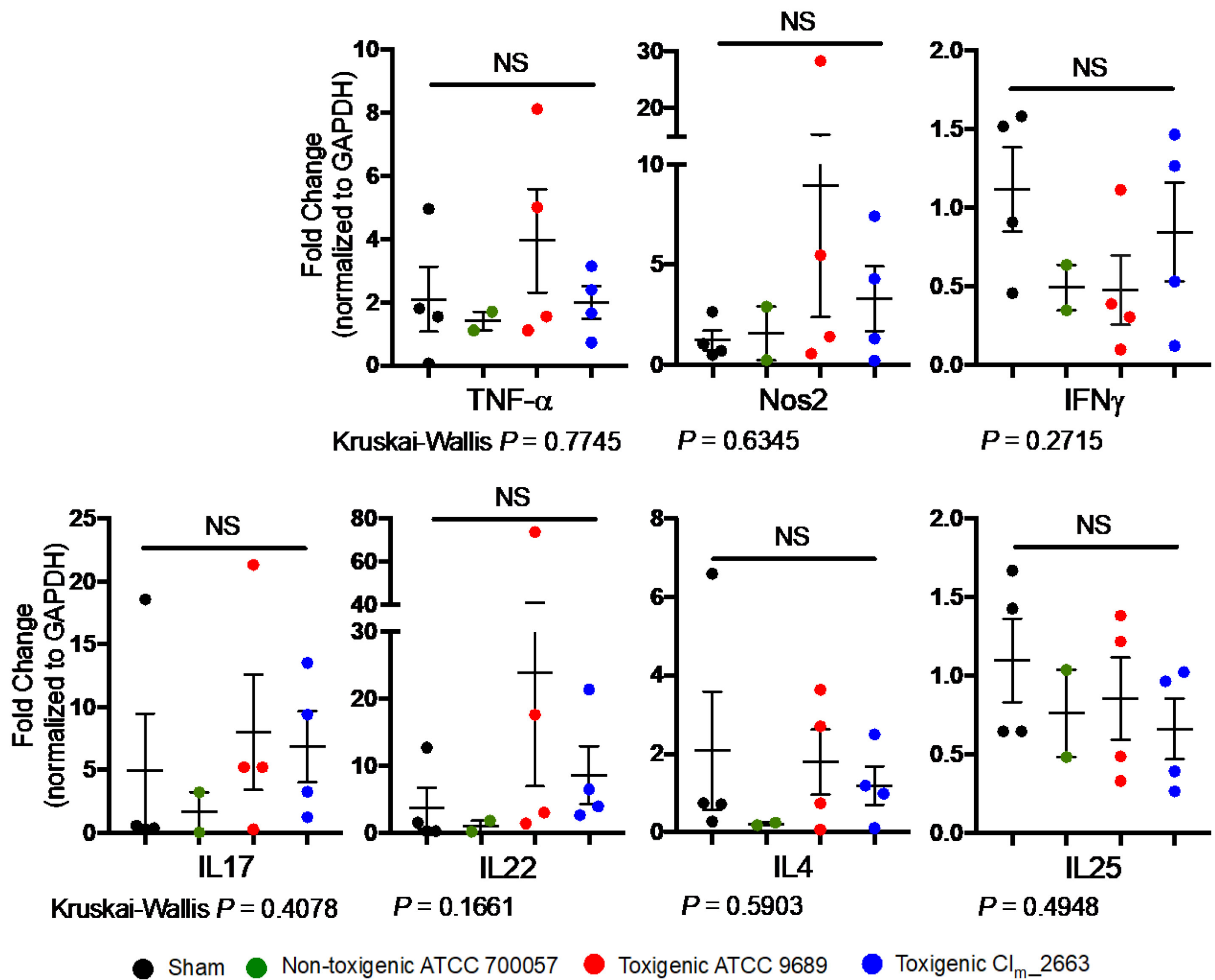


Figure 3.5 Differential cytokine gene expression in the sub-acute phase at 2 weeks after *C. difficile* inoculation in GF wild-type mice and in SPF $Apc^{Min\Delta716/+}$ mice. A. Colonic cytokine gene expression phase at 2 weeks after *C. difficile* inoculation in GF wild-type mice; B. Colonic cytokine gene expression at 2 weeks after *C. difficile* inoculation in SPF $Apc^{Min\Delta716/+}$ mice. TaqMan qPCR targeting cytokine genes including IL-6 (only included in GF mouse experiment), TNF-α, Nos2, IFN-γ, IL-17 (is IL-17A in this figure), IL-22, IL-4, IL-25 were performed using RNA isolated from 0.5 cm colon snips at 2 weeks after inoculation. GF data represent two independent experiments, and SPF data represent one single experiment. The overall *P* value among 4 groups was calculated using Kruskai-Wallis test. The statistical difference between two groups was performed using uncorrected Dunn's test. A *P* value < 0.05 is considered statistically significant.

after inoculation did not show differential gene expression between *C. difficile*-colonized mice and sham that, as noted, did not exhibit either body weight loss (Figure 3.4C) or colitis signs after inoculation (Figure 3.5).

3.4.5 Spatial localization of *C. difficile* in mouse colons and tumors. We sought to explore the spatial localization and the biofilm-forming capability of *C difficile* in the colonic environment of GF wild-type mice and SPF $Apc^{Min\Delta716/+}$ mice at 2 weeks after inoculation. To preserve the colonic mucus layer, 3 cm of distal mouse colons were fixed in Carnoy's solution immediately after dissection, followed by paraffin embedding and sectioning as described in the Methods. *C. difficile* species-specific probe and all bacteria universal probe for the 16S rRNA gene were applied on distal colon sections for FISH hybridization. By confocal microscopy, we visualized *C. difficile* sparsely localizing in the inner mucus layer of the colon while the vast majority populated the luminal space. However, we did not observe a mucus-invasive densely packed bacterial biofilm of *C. difficile* single species in mono-associated GF mice.

To further test whether *C. difficile* in a microbial community acts as a driver bacterium in promoting biofilm formation by recruiting other bacteria to the inner mucus layer, we examined *C. difficile* localization in SPF $Apc^{Min\Delta716/+}$ mice at 2 weeks by FISH as described above. Similar to GF wild-type mice, we visualized a limited number of *C. difficile* and other bacterial cells in the inner mucus layer but did not identify a bacterial biofilm community (Figure 3.7A). The spatial localization of *C. difficile* strains did not vary between the non-toxigenic strain and toxigenic strains. To address whether *C. difficile*

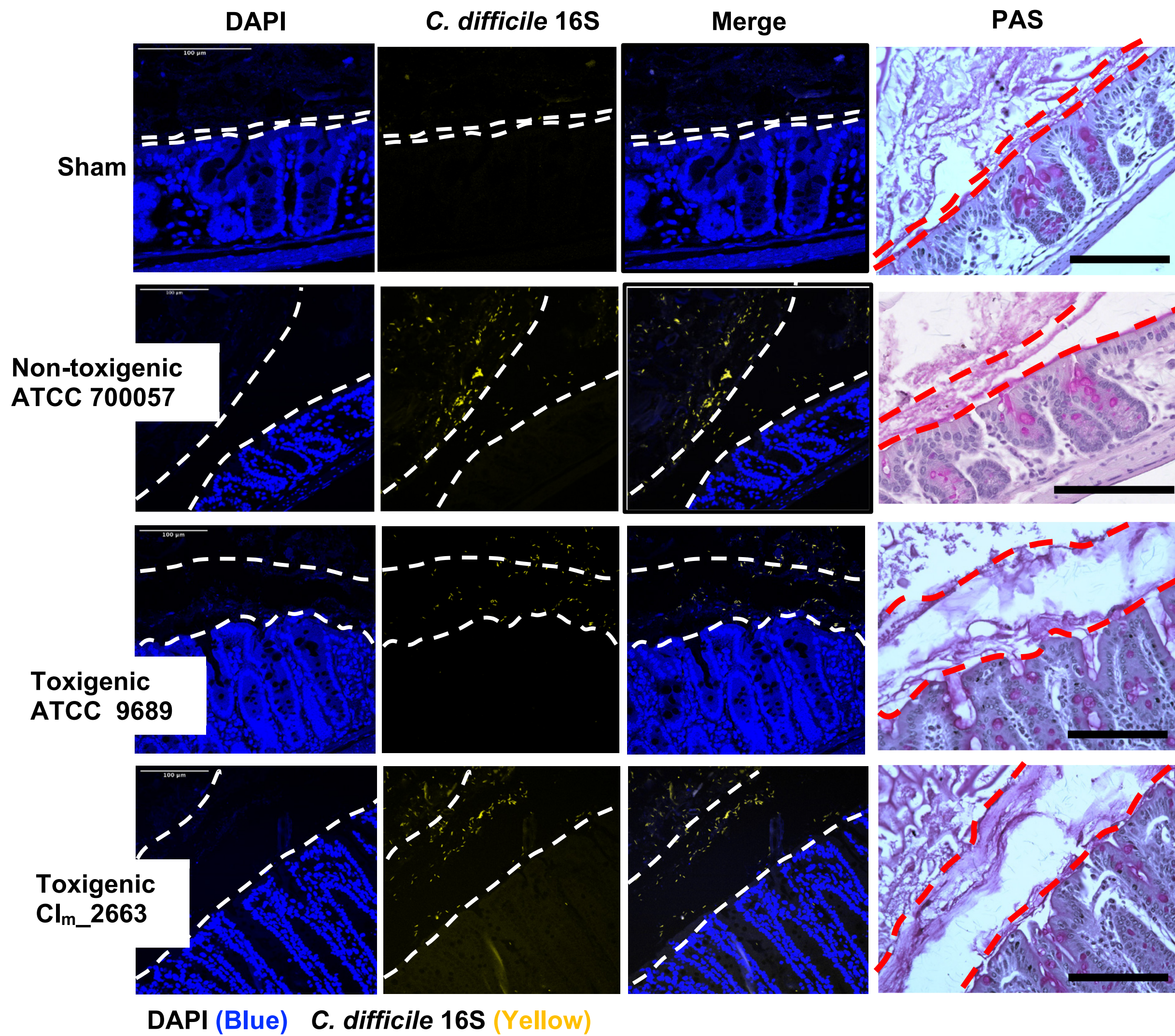


Figure 3.6 *C. difficile* spatial localization in both outer and inner mucus layers in GF wild-type mouse colons at 2 weeks after inoculation. The localization of *C. difficile* strains in GF wild-type mouse colons was identified with *C. difficile* species-specific probe (yellow) for FISH staining, and epithelial nucleus were counterstained by DAPI (blue). Mucus on colon sections was visualized with PAS staining. Scale bars: 100 μm. Data represent two independent experiments.

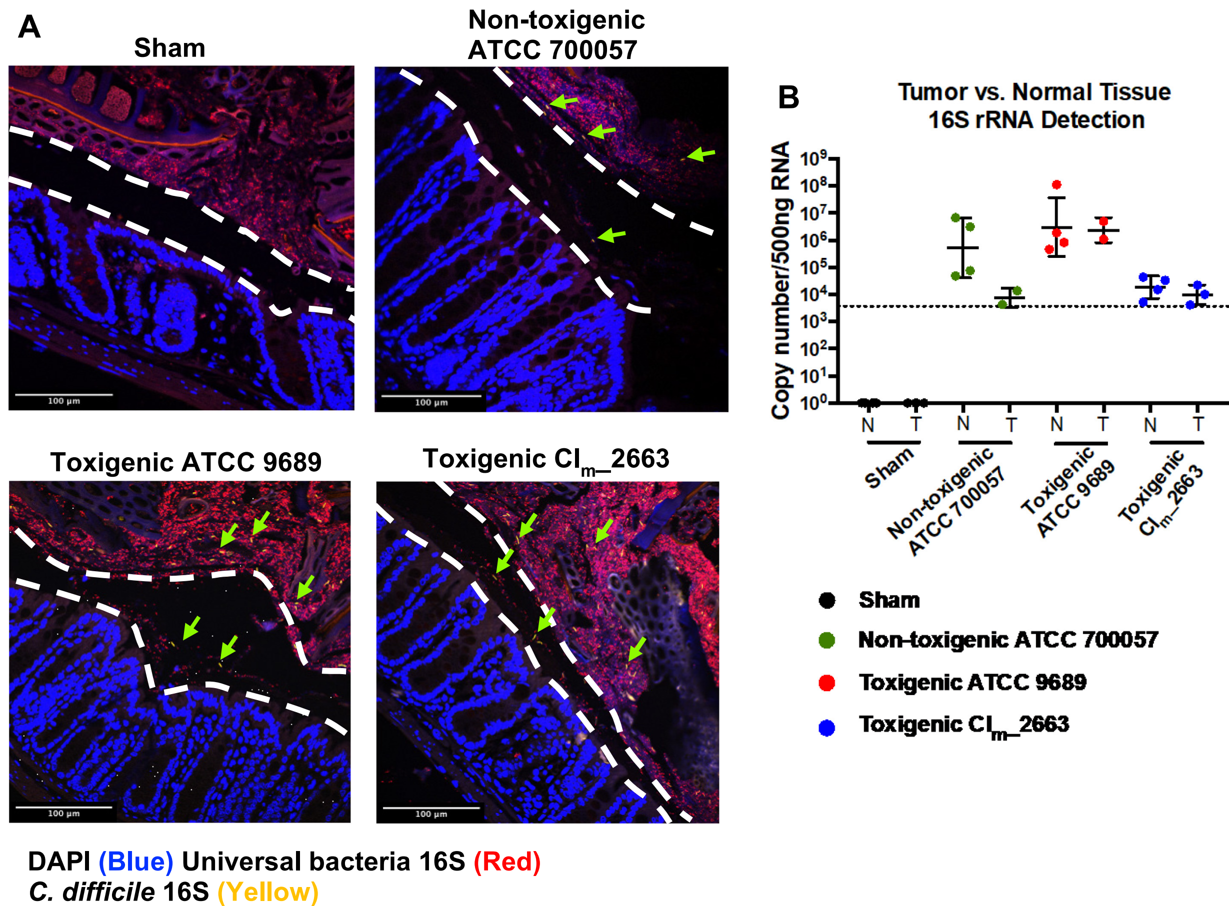


Figure 3.7 ***C. difficile*** **spatial localization in SPF** ***Apc***$^{Min\Delta716/+}$ **mouse colons at 2 weeks and 12 weeks after inoculation.** A. *C. difficile* colonize in both outer and inner mucus layers in SPF *Apc*$^{Min\Delta716/+}$ mice at 2 weeks after inoculation. *C. difficile* localization (green arrows) was identified with the *C. difficile* species-specific probe (yellow) and bacterial universal prole (red) for FISH staining. Epithelial nuclei were counterstained by DAPI (blue). B. Tissue-associated *C. difficile* was quantified by RT-qPCR with RNA isolated from tumors or normal colon pieces of SPF *Apc*$^{Min\Delta716/+}$ mice at 12 weeks after inoculation. Data represent one single experiment with numbers of mice per group: N = 4 (Sham), N = 2 (Non-toxigenic ATCC 700057), N = 4 (Toxigenic ATCC 9689) and N = 4 (CI_m_2663). Images were obtained at 40x magnification. Scale bars: 100 μm.

displays a special tumor-associated niche, we performed RT-qPCR with RNA isolated from tumor or paired normal colon tissues from SPF *Apc*$^{Min\Delta716/+}$ mice at 12 weeks after colonization (Figure 3.7B). The quantification of *C. difficile* copy numbers in tumors did not differ from normal colonic tissues regardless of *C. difficile* infection status.

Colonic *C. difficile* has been reported to alter mucus production and composition in CDI patients (Engevik *et al*. 2014). To further understand the impact of *C. difficile* on the mucus barrier, we utilized our GF wild-type mouse model to avoid the influence of the gut microbiota. We measured mucus depth on PAS-stained colon sections and quantified To further test whether *C. difficile* in a microbial community acts as a driver bacterium in promoting biofilm formation by recruiting other bacteria to the inner mucus layer, we examined *C. difficile* localization in SPF *Apc*$^{Min\Delta716/+}$ mice at 2 weeks by FISH as described above. Similar to GF wild-type mice, we visualized a limited number of *C. difficile* and other bacterial cells in the inner mucus layer but did not identify a bacterial biofilm community (Figure 3.7A). The spatial localization of *C. difficile* strains did not vary between the non-toxigenic strain and toxigenic strains. To address whether *C. difficile* displays a special tumor-associated niche, we performed RT-qPCR with RNA isolated from tumor or paired normal colon tissues from SPF *Apc*$^{Min\Delta716/+}$ mice at 12 weeks after colonization (Figure 3.7B). The quantification of *C. difficile* copy numbers in tumors did not differ from normal colonic tissues regardless of *C. difficile* infection status.

Colonic *C. difficile* has been reported to alter mucus production and composition in CDI patients (Engevik *et al*. 2014). To further understand the impact of *C. difficile* on the mucus

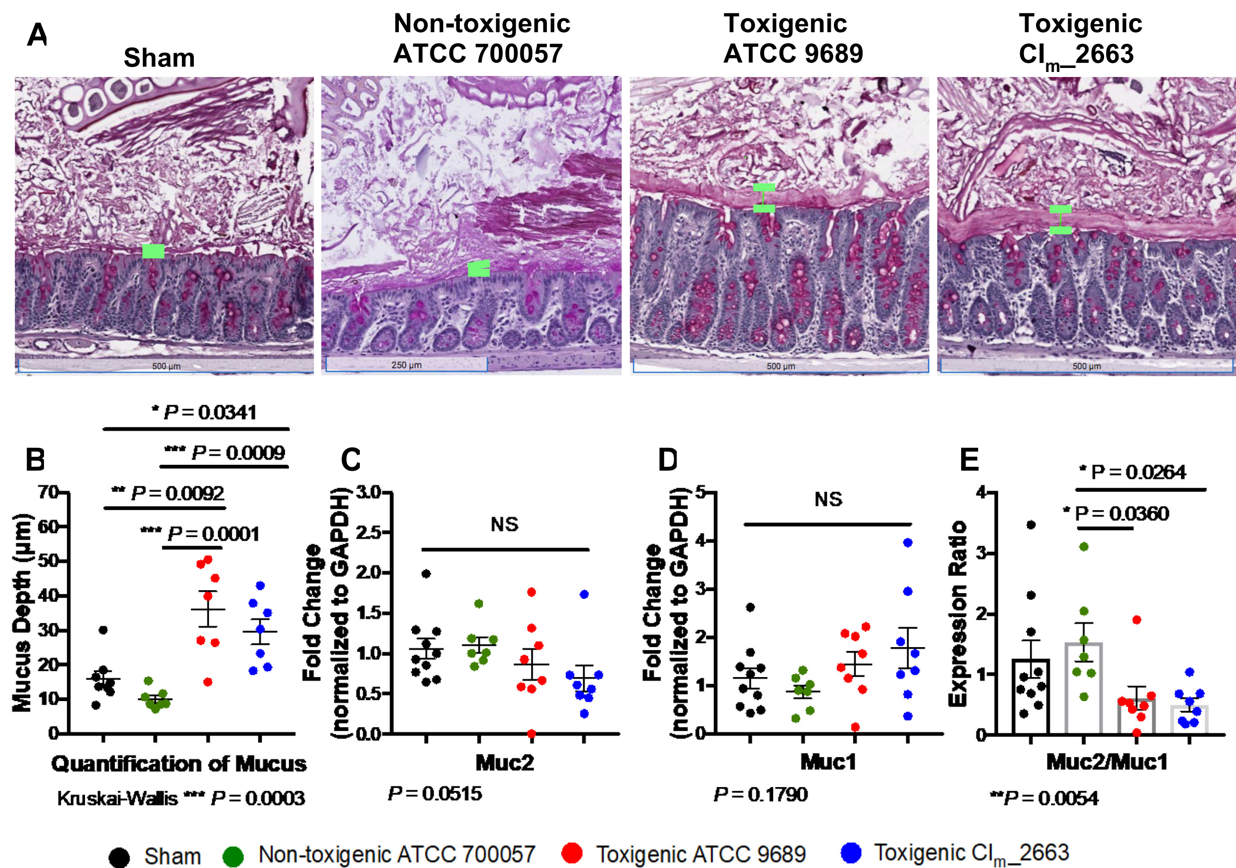


Figure 3.8 Toxigenic *C. difficile* strains induce mucus production in mono-associated GF wild-type mice. A. PAS staining of the distal 3 cm of colons from GF wild-type mice at 2 weeks after inoculation with *C. difficile* strains relative to sham GF mice. Green brackets demarcate the mucus depth (10x magnification, scale bars: 250 μm or 500 μm). B. Comparison of mucus depth among *C. difficile* infection groups. C-E. Muc2 (C), Muc1 (D) gene expression in colonic tissues, and Muc2/Muc1 expression ratio (E). Data of 8 mice per group from Sham, Toxigenic ATCC 9689 and CI_m_2663 groups were collected from two independent experiments, and 8 mice inoculated with non-toxigenic strain ATCC 700057 were from a separate single experiment with additional 2 sham GF mice as control. For mucus depth analysis (A and B), numbers of mice per group: N = 8 (Sham), N = 7 (Non-toxigenic ATCC 700057), N = 7 (Toxigenic ATCC 9689) and N = 7 (CI_m_2663). For gene analysis (C-E), numbers of mice per group N = 10 (Sham), N = 7 (Non-toxigenic ATCC 700057), N = 8 (Toxigenic ATCC 9689) and N = 8 (CI_m_2663). The overall *P* value among 4 groups was calculated using Kruskai-Wallis test. The statistical difference between two groups was performed using uncorrected Dunn's test. A *P* value < 0.05 is considered statistically significant.

barrier, we utilized our GF wild-type mouse model to avoid the influence of the gut microbiota. We measured mucus depth on PAS-stained colon sections and quantified mucus-related gene expression using qPCR at 2 weeks after inoculating *C. difficile* strains into GF wild-type mice. As shown in Figure 3.8A and B, mono-colonization of toxigenic *C. difficile* strains increased mucus depth in *C. difficile* mono-associated GF mice as compared with sham and non-toxigenic *C. difficile*-infected GF mice. Despite similar expression of the mucus-related genes, Muc2 and Muc1, across groups, toxigenic *C. difficile* strains decreased the expression ratio of Muc2/Muc1. These results suggest that in response to toxigenic *C. difficile* strains and *C. difficile* toxins, the mucus composition and secretion by colonic epithelial cells may be modulated.

3.4.6 Phylogenetic analysis of *C. difficile* strains. To characterize the *C. difficile* strains used in our study, we performed whole-genome sequencing of the human CRC-associated strain CI_m_2663 together with strain ATCC 700057 for which a genome sequence has not been published. Referring to publicly available *C. difficile* whole genome sequences, we performed phylogenetic analysis. The result showed that CRC CI_m_2663 isolate was in a different clade from the toxigenic strain ATCC 9689 or non-toxigenic *C. difficile* strain ATCC 700057 (Figure 3.9). The comparison of *tcdA* and *tcdB* toxin sequences suggested that *tcdB* gene regions, harboring 98.1% matched contigs, are nearly identical between CRC CI_m_2663 isolate and toxigenic strain ATCC 9689, whereas *tcdA* genes differ with only 78.6% matched contigs. At present, we cannot specifically test the biological activity of toxin A verses toxin B from *C. difficile*

strains ATCC 9689 and CRC CI_m_2663 because of the structural and functional overlap between these two toxins. However, we plan to create toxin-specific knock-out strains that, in the future, will enable us to discern if the lower homology of the toxin A genes is biologically relevant.

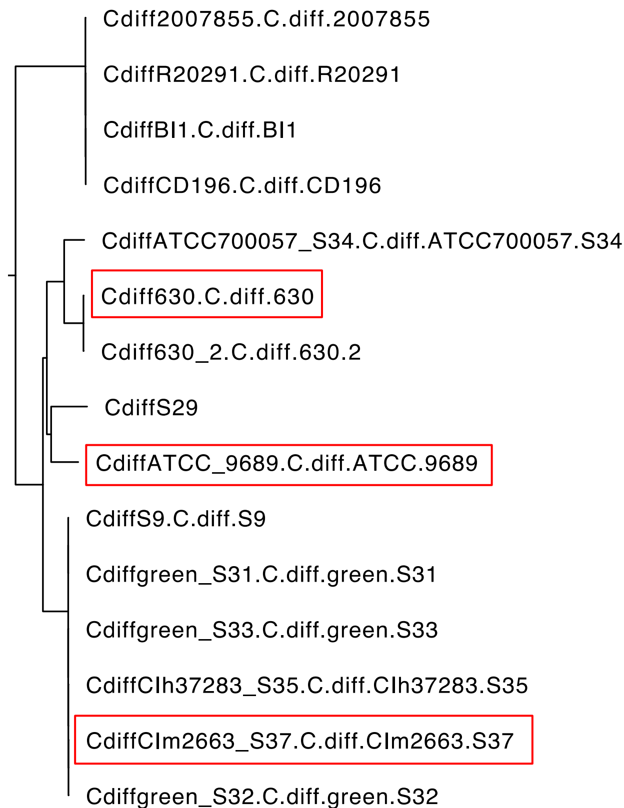


Figure 3.9 Whole genome sequence comparison among strains included in this study. Phylogenetic analysis of *C. difficile* strains CRC CIm_2663 and non-toxigenic ATCC 700057, in reference to toxigenic strain ATCC 9689, each highlighted within a red box.

3.5 Discussion

A mouse model of *C. difficile* infection or colonization should ideally be standardized to generate reproducible results and resemble the pathogenesis of human diseases. Our chronically colonized SPF *Apc*$^{Min\Delta716/+}$ mice established a new mouse model to examine the potential long-term impact on disease pathogenesis in *C. difficile* carriers. In fact, the biologic impact of persistent *C. difficile* colonization in humans is unknown. Further, our data raise the hypothesis that the patient with a congenital or an acquired *APC* gene mutation may face an increased risk of colon tumor development over time if asymptomatically colonized with toxigenic *C. difficile* strains. Determining how commonly pro-tumorigenesis is driven by *C. difficile* strains in human populations requires further study. Importantly, *C. difficile* strains may possess variable disease potential as we see our CRC CI_m_2663 isolate tends to be more tumorigenic than toxigenic ATCC 9689 in SPF *Apc*$^{Min\Delta716/+}$ mice; further, some *C. difficile* strains, including toxigenic *C. difficile* strains, may not be pro-tumorigenic. Emerging evidence suggests that non-toxigenic virulence factors including cell wall proteins, adherence factors and metabolic products may influence disease severity in mouse models (Awad *et al*. 2015). Genome mining and experimental work with differing *C. difficile* strains may even discover new tumorigenic virulence determinants.

Antibiotics modify the metabolic activity and the colonization resistance of altered microbiota, thereby increasing host susceptibility to *C. difficile*. In addition to multiple antibiotic cocktail regimens, vancomycin priming treatments for *C. difficile* mouse models range from a single administration to daily administration over 5 days with widely variable

doses to yield acute *C. difficile* colitis mouse models. In order to chronically colonize *Apc*$^{Min\Delta716/+}$ mice with *C. difficile*, we adjusted previous antibiotic regimens and utilized vancomycin and gentamicin pre-treatment followed by continuous gentamicin in drinking water to facilitate sustained and non-lethal *C. difficile* colonization for 12 weeks in the SPF *Apc*$^{Min\Delta716/+}$ mice. Gentamicin has no activity against *C. difficile*, when given orally, y*et al*ters the mouse microbiota sufficiently to be permissive to chronic *C. difficile* colonization. In both pilot studies and later our refined studies, the non-toxigenic *C. difficile* strain was less likely to initiate or sustain persistent colonization under our antibiotic therapy approach (Figure 3.2). Interestingly, we have not yet successfully colonized SPF wild-type mice with *C. difficile* under the adjusted antibiotic priming. The reason why SPF wild-type mice were resistant to *C. difficile* colonization still requires study, however, the genetic difference between wild-type and *Apc*$^{Min\Delta716/+}$ mice may impact the gut microbial composition, host-microbiota metabolism interactions such as primary bile acid availability or influence the protein or other molecule expression of the colon epithelial cells, leading to differential susceptibility to *C. difficile* colonization. Similarly, the more consistent and persistent colon colonization by two distinct toxigenic *C. difficile* strains (Figure 3.9) suggests that either the toxins and/or other adherence determinants augment *C. difficile* mucosal colonization potential, an observation of possible importance to chronic asymptomatic or symptomatic *C. difficile* colonization in humans. Further, it may be that human with congenital or acquired *APC* gene mutations may harbor a gut and/or mucosal microbiota prone to *C. difficile* colonization. These data together highlight the significance and importance to future research of multi-factor assessment criteria

including genetics, microbiota and/or host tissue histology in colonoscopy screening for CRC.

GF mice are a valuable experimental tool to investigate host–bacterial interactions in an environment devoid of competitive interactions from gut microbiota. In Figure 3.3 and Figure 3.4, we show distinct disease course and corresponding differential cytokine gene expression at 2 weeks in GF wild-type mice relative to SPF *Apc*$^{Min\Delta716/+}$ mice at the same time point. This may be due to the impaired and naïve mucosal and/or systemic immune system in GF mice. Importantly, acute inflammation induced in CDI murine models (Buonomo *et al*. 2016; Abt *et al*. 2015; Behnsen *et al*. 2014) has been extensively studied and results suggest that IL-17A, IL-22, IL-25 together with neutrophils, eosinophils and innate lymphoid cells, ILC3, are important effectors in host defenses and the inflammatory pathogenesis. Given *C. difficile* infection in mice typically induces mucosal inflammation at 1 to 4 days, we chose 2 weeks after inoculation when acute inflammation and epithelial damage was expected to be resolving based on histology to characterize the consequences of persistent colonization that may contribute to colon tumor development. Of note, study of a mono-associated mouse model (i.e., inoculation into germ-free mice) strongly indicated that *C. difficile* induces early mucosal inflammation at 2 weeks marked by upregulated macrophage, Th17 cell activities and innate lymphoid cell functions including upregulated expression of TNF-α and NOS2, IL-17A and IL-22. These results, despite not being replicated at this time point in SPF mice, provide potential clues to further assess the pro-tumorigenic environment with additional chronic colonization experiments that will be described in the next chapter.

We did not detect that *C. difficile* strains assemble biofilms on FISH-stained colon sections in SPF *Apc*$^{Min\Delta716/+}$ mice at 2 weeks preceding tumor initiation. Further *C. difficile* 16S rRNA qPCR analysis of normal colonic tissues and tumors from SPF *Apc*$^{Min\Delta716/+}$ mice at 12 weeks showed that *C. difficile* does not aggregate specifically in tumor areas, consistent with the hypothesis that chronic *C. difficile* colonization likely precedes onset of tumorigenesis. This timeline is consistent with *C. difficile* being a promoter of colon tumor development. Overall, our results encourage us to further consider secreted molecules, particularly toxins, to explore the mechanisms by which *C. difficile* induces tumors, a topic that will be discussed in the next chapter. Similarly, we found that even GF mice were not permissive to a mono-species *C. difficile* colon mucosal biofilm but rather, our gene expression studies in GF mice suggest that toxigenic *C. difficile* promotes mucus secretion and an alteration of mucus type composition. These results suggest that toxigenic, but not non-toxigenic, *C. difficile* alters barrier function that may contribute to chronic colonization and inflammation in the mucosa. Normal colonic mucus is primarily composed of secreted MUC2 mucin and cell membrane-associated mucins such as MUC1. Mucins are highly glycosylated proteins and altered mucin expression and glucosylation have been observed in human colon cancer specimens. Muc2$^{-/-}$ mice display aberrant crypts and adenomas in the small intestine (Velcich *et al*. 2002). Our data suggest that toxigenic *C. difficile* strains may alter mucin gene expression of Muc2/Muc1, however, whether the mucin proteins play a role in colon tumorigenesis in *Apc*$^{Min\Delta716/+}$ mice and humans remain to be investigated. It is possible that the mucus alteration stimulated by *C. difficile* is a host defense mechanism.

One limitation of these experiments is that the non-toxigenic strain ATCC700057 is not an isogenic mutant generated from either toxigenic strains ATCC9689 or CI_m_2663. Hence, we cannot exclude that other potential *C. difficile* virulence factors than toxin A and B may also contribute to the disease pathogenesis observed from these two strains. Further, additional time course experiments and studies of genetically and clinically divergent toxigenic and non-toxigenic *C. difficile* strains would be optimal.

3.6 Conclusion

Toxigenic *C. difficile*, particularly our CI_m_2663 strain isolated from the colonic mucosa microbiota of one CRC patient, induces colon tumorigenesis over 12 weeks in $Apc^{Min\Delta716/+}$ mice and we propose that *C. difficile* toxins may be involved in the tumorigenesis mechanisms in humans. *C. difficile* has the capability to establish a mucus-associated niche in both GF wild-type and SPF $Apc^{Min\Delta716/+}$ mice at 2 weeks. but fails to form mature biofilms as either single species or polymicrobial communities, respectively. Further, *C. difficile* induced colon tumor development in SPF $Apc^{Min\Delta716/+}$ mice is not accompanied by tumor-associated aggregates or biofilms. The specific virulence properties of the CRC isolate CI_m_2663 and ATCC 9689 including the toxin pathogenicity loci and non-toxin virulence factors remain to be identified and characterized with more detailed genomic and experimental analysis.

Chapter 4

Persistent *C. difficile* toxin production induces chronic mucosal inflammation and modifies epithelial biology that correlates with increased colon tumorigenesis in *Apc*$^{Min\Delta716/+}$ mice

Abstract

Emerging evidence of certain bacterial species inducing tumorigenesis in animal models supports the association between specific pathogenic bacteria and human carcinogenesis. The microbial-dependent tumorigenic mechanisms include bacterial virulence factors, inflammation and functional and/or genetic alterations in epithelial cells. Our results suggest that toxigenic *C. difficile*-induced colon tumorigenesis in SPF *Apc*$^{Min\Delta716/+}$ mice depends on persistent *C. difficile* toxin production. The persistent toxin production during *C. difficile* chronic colonization facilitates the development of low-grade mucosal inflammation that displays predominant infiltration of macrophages in colonic mucosa along with differential cytokine expression in tumor environment. We propose these features predispose normal epithelial cells to a pro-inflammatory, pro-tumorigenic environment that fosters persistent epithelial hyperplasia and the sequential tumor progression.

Introduction

A substantial body of studies illustrates that chronic inflammation predisposes a healthy

individual to cancer by re-shaping the tissue microenvironment and epithelial biology over time (Gagliani *et al*. 2014; Arthur *et al*. 2012). This realization is typically represented by the association between chronic inflammatory bowel diseases and the increased risk of colorectal cancer (Swidsinski *et al*. 2009; Grivennikov *et al*. 2012). Existing data demonstrate that the microbiota associated with colon cancer contributes to colon tumorigenesis dependent on biologic mechanisms driven by microbial virulence factors including inflammation, DNA damage and pro-oncogenic signaling activation (Gao *et al*. 2015; Goodman and Gardner 2018; Goodwin *et al*. 2011; Wu, S. *et al*. 2009; Rubinstein *et al*. 2013; Arthur *et al*. 2012). However, the mechanisms by which *C. difficile* may cause colon tumorigenesis in mouse models and humans remains unexplored.

Thus far, outstanding evidence shows that *C. difficile* toxin A and toxin B are the major virulence factors mediating the pathogenesis of *C. difficile*-induced acute colitis that is predominantly characterized by inflammation due to innate immune responses (Awad *et al*. 2015; 1995; Lyras *et al*. 2009). These toxins are transported into the colonic epithelial cells and are known to glucosylate small Rho and ras family GTPases to disrupt the actin cytoskeleton and ultimately impair epithelial integrity. Further, toxin A and toxin B induce the secretion of inflammatory factors including cytokines, chemokines and reactive oxygen mediators that trigger a mucosal inflammatory cascade, such as toxin A and toxin B directly activating the epithelial cells, tissue resident macrophages, dendritic cells (DCs) to produce epithelial IL-8/CXCL-8, monocyte chemoattractant protein 1 (MCP-1), macrophage-derived TNF-α, IL-8/CXCL8, IL-1β, DC-derived IL-1β, IL-6 and IL-23 (Chandrasekaran and Lacy 2017; Carter *et al*. 2010; Buonomo and Petri 2015; Solomon

2013; Sun and Hirota 2015; Saleh and Petri 2020). These cytokines and chemokines play an important role in recruiting neutrophils to the colonic mucosa that is recognized as the inflammatory hallmark of CDI-induced acute inflammation (Kelly,Becker, *et al*. 1994; Kelly,Keates, *et al*. 1994; Linevsky *et al*. 1997; Kelly and Kyne 2011; Sun and Hirota 2015). Furthermore, increasing data suggest that 3 types of Innate lymphoid cells (ILCs) provide early protection in various ways during CDI (Geiger *et al*. 2014; Buonomo and Petri 2015; Abt *et al*. 2015). ILC1-derived IFN-γ, Nos2, and ILC3-derived IL-17A, IL-22 are key cytokines predominantly upregulated in response to CDI, and synergistically play protective roles from severe CDI disease (Abt *et al*. 2015; Hasegawa *et al*. 2014; 2012). Interestingly, independent studies found that type 2 cytokines of IL33 (Frisbee *et al*. 2019) and IL25 (Buonomo *et al*. 2016) are suppressed during human and/or murine CDI. The downstream eosinophils contribute to maintaining epithelial integrity likely regulated by ILC2 (Frisbee *et al*. 2019; Buonomo *et al*. 2016). The adaptive immune responses, particularly mucosal Th17 cell functions, have been largely understudied (Saleh and Petri 2020) as compared with innate immunity in the context of CDI. IgA or IgG antibodies against *C. difficile* toxins provide long-lived protection from recurrent CDI in mouse models (Johnston *et al*. 2014) ; however $CD4^+$ T cells are not required for IgA class switching. It remains unclear whether $CD4^+$ T cells or the Th17 subs*et al*one contribute to CDI protection or immunopathology in the long term (Ryan *et al*. 2011; Maseda *et al*. 2019). Overall, on balance, pro-inflammatory responses result in acute colitis marked by extensive tissue damage, fluid secretion, bleeding and/or pseudomembranous lesions. Moreover, many human CDI cases develop relapse or recurrence potentially triggering chronic colitis that is less well-studied in the CDI field. Some studies in mouse models

report that colonization with *C. difficile* causes chronic inflammation lasting up to 1 month after the acute CDI symptoms resolve (Buffie *et al*. 2012). However, the possibility that CDI transforms to chronic colon inflammation and the consequence of this putative chronic inflammation on epithelial cell biology still needs to be characterized, particularly in humans.

In addition to the chronic colon inflammation that *C. difficile* toxins may induce, the potential impact on epithelial cells that are persistently exposed to toxin A and toxin B in a chronic fashion has not been well studied. Current studies have only focused on the acute effects of toxins on multiple aspects of epithelial cell biology such as disruption of cell cytoskeleton and cell-cell tight junctions, induction of apoptosis and necrosis (Kuehne *et al*. 2010; Chandrasekaran and Lacy 2017). Notably, limited data suggest toxin B, instead of toxin A, might be more associated with colon tumorigenesis, although research shows controversial results. On one hand, an earlier study found that toxin B activates epidermal growth factor receptor (EGFR)-ERK-MAP signaling pathway in human colonic epithelial cells (Na *et al*. 2005), suggesting that toxin B may contribute to promoting tumorigenesis. The more recent studies, on the other hand, suggest that toxin B competitively binds to Wnt receptor on cell membrane and effectively blocks Wnt/β-catenin signal activity (Chen, P. *et al*. 2018; Tao *et al*. 2016), suggesting an anti-tumor activity of toxin B. A newly published study reported that *C. difficile* toxin B reduced the extracellular domain of the cell-cell adherens junctional protein, E-cadherin, at 48 hours in infected mouse colons (Mileto *et al*. 2020), and demonstrated that colonic epithelial cells upregulate gene expression of stem cell markers and Wnt signaling targets. These

observations suggest that *C. difficile* toxin B may activate the Wnt/β-catenin signaling pathway as β-catenin binds to the intracellular domain of E-cadherin and is released upon E-cadherin cleavage. However, more studies are needed to determine whether *C. difficile* toxin B promotes colon tumor formation via altered Wnt/β-catenin signaling.

Additionally, Chumbler and colleagues (Chumbler *et al*. 2016) demonstrated that at higher concentrations (100pM or above), toxin B initiates the assembly of the NADPH oxidase (NOX) complex on endosomes leading to aberrant production of endosomal reactive oxygen species (ROS) in cultured epithelial cells. It was also suggested that, unlike toxin B, toxin A does not enhance ROS production, but causes a glucosylation-dependent apoptosis at all concentrations. These data raised the hypothesis that toxin B might induce DNA damage by enhancing ROS response. However, another study (Fettucciari *et al*. 2018) demonstrated that toxin B induces early and persistent DNA damage independent of ROS pathways in enteric glial cells. Whether the toxin B could provoke a robust ROS production *in vivo* and/or induce DNA damage to possibly contribute to tumorigenesis is unclear.

Cytotoxicity assays indicate that Vero and HT29/C1 cells are both susceptible to toxin A and toxin B but Vero cells are more sensitive to toxin B and HT29/C1 cells are more sensitive to toxin A. However, whether toxin A or toxin B is more essential to disease severity *in vivo* remains to be elucidated. One study (Lyras *et al*. 2009) showed that a *tcdA*$^{+}$*tcdB*$^{-}$ strain is completely avirulent in mice whereas in another study, *tcdA*$^{+}$*tcdB*$^{-}$ and *tcdA*$^{-}$*tcdB*$^{+}$ strains were similarly virulent (Kuehne *et al*. 2010). The discrepancy

between these two studies may be accounted for by the different methods used to make the mutant bacterial strains, which resulted in different cytotoxicity and toxin titers in bacterial culture between the mutants. Neither of these two studies measured toxin levels or biology *in vivo* which may be critical to determine the significance of toxin A versus toxin B.

Hence, in this study, after assessing the association of toxigenic *C. difficile* strains with chronic inflammation and promotion of colonic tumorigenesis in 12-week colonized *Apc*$^{Min\Delta716/+}$ mice (Chapter 3), we sought to further characterize the chronic inflammation, cell proliferation and DNA damage associated with longitudinal toxin production *in vivo*. We hypothesized that these changes are important for initiating aberrant crypt foci and microadenomas, or to promote later tumor growth in *Apc*$^{Min\Delta716/+}$ mice.

4.3 Materials and Methods

4.3.1 *C. difficile* strains and mouse experiment. In this chapter, we analyzed the mouse specimens including feces, colonic snips and tumors, and colon sections from 12-week tumor experiments with SPF *Apc*$^{Min\Delta716/+}$ mice as detailed in Materials and Methods 3.3.5. *C. difficile* strains used in those experiments included non-toxigenic strain ATCC 700057, toxigenic strain ATCC 9689, CRC-associated isolate CI_m_2663. In addition, we also evaluated tumorigenesis in SPF *Apc*$^{Min\Delta716/+}$ mice inoculated with *C. difficile* strain wild-type 630Δ*erm* and isogenic *tcdA*$^{+}$*tcdB*$^{-}$ mutant (see Table 3). Strain *tcdA*$^{+}$*tcdB*$^{-}$ 630Δ*erm* (*tcdB* knockout strain) was originally constructed on the background of wild-type 630Δ*erm* by Dr. Dena Lyras in Australia (Lyras *et al*. 2009). These isogenic strains were

generously provided by Dr. Borden Lacy at Vanderbilt University with Dr. Lyras' kind permission.

4.3.2 Cell Culture. Vero and HT29/C1 cells were maintained in DMEM-high glucose (Dulbecco's modified Eagle's medium with 4.5g/L glucose, Sigma) supplemented with 10% (v/v) FCS (fetal calf serum; Sigma) and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO2 until confluence. To pass the cells, the cells were dissociated using trypsin-EDTA (0.05%, Thermo Fisher Scientific) and adjusted to the desired cell concentration of 2 x 10^6/ml with complete cell culture medium stated above. We seeded 96-well plates with 10^5 Vero cells/well 1-2 days prior to performing the cytotoxicity assay or 10^5 HT29/C1 cells/well 3 days prior to cytotoxicity assay.

4.3.3 Cytotoxicity Assay. 0.02-0.10 g fecal pellet (1 g/ml, e.g., 0.1 g = 0.1 ml) was suspended in 9 volumes of sterile PBS (e.g., 0.9 ml PBS to 0.1 g stool) to make a fecal homogenate (0.1 g/ml) that is 1:10 dilution of the original feces. Stool homogenates were centrifuged at 4,000g, 4°C for 10 min, and the supernatants were diluted in a tenfold series using sterile PBS. 10 µl of each dilution was added onto the Vero cells (African green monkey kidney cell line, ATCC® CCL-81™) and HT29/C1 cells (human colon carcinoma cell line, kindly provided by Dr. Daniel Louvard) at 70%-100% confluence cultured in DMEM supplemented with 10% (v/v) FCS and penicillin/streptomycin on 96-well plates. Cell rounding was scored from each dilution after 24h incubation, and the highest dilution with 100% cell rounding that indicated complete cytopathic effects (CPE) was recorded as the end-point toxin titer. Toxin titers are expressed as the $\log_{10}$

(reciprocal of the highest dilution factor to display 100% cell rounding)/gram stool). All assays were carried out in triplicate.

4.3.4 Toxin B ELISA. *C. difficile* toxin B ELISA was carried out utilizing the Fecal *C. difficile* Toxin B qualitative ELISA Assay Kit (Eagle Biosciences). The stool homogenates used in this experiment were previously made for fecal *C. difficile* colonization measurements presented in Chapter 3 and the cytotoxicity assays presented in this chapter, to maintain consistency between these assays for comparison and quality control purposes. Since this ELISA Kit was designed for human fecal sample analysis, stool resuspension was adapted from a 1:5 dilution in the protocol to 1:20 dilution with mouse stools of lower weight. We followed the procedure manual for all the other steps. In brief, we centrifuged the stool homogenates (1:10 dilution of the original stools) at 10,000 g, 4°C for 5 min and took 125 μl of supernatant from each sample into a new tube. 25 μl of 10-fold concentrated fecal sample extraction buffer and 100 μl of distilled water was added to make a final volume of 250 μl; the extracted sample was now at a 1:20 dilution from the original stool. We pipetted 100μl of controls and extracted mouse stool samples into the microwells coated with Toxin B monoclonal antibody, and incubated the microwell plate at room temperature, static, for 1 hour. After 5 washes following each incubation, 100 μl of Toxin B Tracer Antibody and ELISA HRP Substrate were added into each of the reactions for toxin B binding and development. The positive wells turned yellow visually, and we stopped the development after 7 min incubation with HRP Substrate. The optical absorbance was read at 450 nm using a microplate reader, and toxin B level was expressed as Optical Density (OD_{450}).

4.3.5 Histopathologic assessment. Mice were necropsied at the end of 12-13 weeks after inoculation with *C. difficile* strains or sham inoculation. We flushed out the colon contents with sterile PBS and cut open the colons longitudinally to examine colonic tumors as described in Chapter 3. We fixed the colons in 10% neutral buffered formalin solution overnight followed by transferring into 0.9% saline supplemented with 0.3% Na_3N. For histopathology, the colons were Swiss-rolled for paraffin-embedding, sectioning (4 μm) and hematoxylin and eosin (H&E) staining by Oncology Tissue Services Core Facility at the Johns Hopkins University. Histological scoring of inflammation, epithelial damage, hyperplasia and crypt organization was performed blindly by S.W. Criteria for histological evaluation is detailed in Table 4.

Table 4. Histopathological scoring criteria on mouse colon sections

Score	Inflammatory infiltration	Epithelial damage	Hyperplasia	Crypt Alteration
0	normal	none	normal	normal
1	minimal multifocal in mucosa	minimal superficial epithelial damage	minimal	minimal
2	moderate multifocal, submucosal involvement, crypt abscesses	moderate multifocal superficial damage, few exfoliated colonocytes in lumen	mild	mild
3	severe multifocal mucosal infiltration with greater submucosal involvement	severe multifocal epithelial damage (ulcers), may have early pseudomembrane formation	moderate	moderate
4	marked mucosa and submucosal infiltration, often transmural	same as 3 with well-developed pseudomembrane or ulcer	marked	marked

References: (Theriot *et al*. 2011); (Erben *et al*. 2014)

4.3.6 Immunohistochemistry (IHC) staining. Mouse colons were fixed in 10% formalin or Carnoy's solution followed by paraffin-embedding and sectioning as described in the Chapter 3. Unstained colon sections were subjected to immunohistochemistry (IHC) staining using the following antibodies: anti-Ki-67 (Catalog #NCL-Ki-67p, Leica

Table 5. Gene List for RT-qPCR analysis	
Cytokine	**ID of TaqMan™ Gene Expression Assay**
IL-6	Mm00446190_m1
Tnfa	Mm00443258_m1
Nos2	Mm00440502_m1
Ifng	Mm01168134_m1
IL-17a	Mm00439618_m1
IL-22	Mm00444241_m1
IL-25	Mm00499822_m1
IL-4	Mm00445259_m1
IL-13	Mm00434204_m1
Tgfb1	Mm01178820_m1
Reg3g	Mm00441127_m1
Mucin genes	
Muc1	Mm00449604_m1
Muc2	Mm00458293_g1
Wnt signaling	
Lgr5	Mm00438890_m1
Axin2	Mm00443610_m1
CD44	Mm01277157_m1
CCND1	Mm00432359_m1

Biosystems, USA), anti-Phospho-Histone H_2AX (Catalog #9718, Cell Signaling, USA), anti-CD3 (Catalog #99940, Cell Signaling, USA), anti-Ly-6G (Catalog #87048, Cell Signaling, USA), and anti-F4/80 (Catalog #70076, Cell Signaling, USA). The slides were dewaxed with xylene and rehydrated through an ethanol serial gradient as described in Chapter 3. The rehydrated colon tissue sections were treated with 3% hydrogen peroxide to abolish endogenous peroxidase activity and steamed in sodium citrate buffer for antigen retrieval. Then, 5% goat serum was applied for 60 min to saturate non-target binding sites, followed by incubation with the primary antibodies at 4°C overnight. The next day, the slides were washed with TBST buffer (Tris-Buffered Saline 0.1% TWEEN ®20, Sigma) and incubated with a second antibody (anti-rabbit IgG, Abcam) for 30 min at room temperature. The slides were developed with DAB (3,3'-Diaminobenzidine, Abcam)

reagent, followed by TBST washes, hematoxylin counterstain, dehydration through an ethanol gradient and mounting with coverslips. For the quantification of Ki-67+ or γ-H_2AX+ cells, 4 or 5 randomly selected areas of normal distal colon demonstrating well-oriented crypts from each section were viewed and counted at 20x or 40x magnification using Image J. Immune cell infiltration of tumors or normal regions in distal colons were quantified using Halo platform (Indica Labs) in the Tumor Microenvironment Lab, Johns Hopkins University School of Medicine.

4.3.7 Tissue RNA extraction, cDNA synthesis, and RT-qPCR. Total RNA from 0.5 cm normal colon tissue or dissected tumors was extracted using the Direct-Zol™ RNA Miniprep kit (Zymo Research) as detailed in Materials and Methods 3.3.7. We used 500ng or 1µg of RNA for cDNA reverse transcription using the High-Capacity RNA-to-cDNA Kit (Thermo Scientific). The target genes evaluated by the TaqMan™ Gene Expression Assay (FAM) are listed in Table 5. The relative mRNA quantities were expressed using a standard ∆∆CT method that calculated fold-changes and were normalized to mouse GAPDH, a housekeeping gene.

4.3.8 Statistical Analysis

Statistical analyses were conducted with GraphPad Prism software version 7.0. Numerical data without a normal distribution were expressed as means ± SE, and differences between two groups were evaluated using Mann-Whitney U test. For multiple group comparisons, statistical analysis was performed with nonparametric Kruskal-Wallis test followed by uncorrected Dunn’s test for each two-group comparison as we

considered each experimental group as independent from others. Differences at $P < 0.05$ were considered statistically significant.

4.4 Results

4.4.1 Persistent *C. difficile* toxin production correlates with colon tumor counts. In Chapter 3, we demonstrated that toxigenic *C. difficile* strains, particularly the human CRC-associated *C. difficile* isolate CI_m_2663, enhanced colon tumor formation. To address the question whether CRC-associated strain CI_m_2663 displays differential virulence features to enhance colon tumorigenesis, we first tested if the CRC-associated strain CI_m_2663 produces more toxins. The longitudinal fecal toxin A and toxin B levels were measured at multiple time points (week1, week3 or 4, week8 and week12) in *C. difficile* colonized $Apc^{Min\Delta 716/+}$ mice utilizing the *in vitro* cell cytotoxicity assays with Vero cells and HT29/C1 cells. Although each of the two cell lines is susceptible to both toxin A and toxin B, previous studies have shown that HT29/C1 cells are more sensitive to toxin A and Vero cells are more sensitive to toxin B when the cross activities are diluted out properly. After treatment with fecal supernatants, the cell rounding effect on Vero cells modestly suggested that CRC-associated strain CI_m_2663 may secrete higher titers of toxin A and toxin B than the toxigenic ATCC 9689 strain until 4 weeks after colonization (Fig. 4.1A. $P = 0.0044$), even when fecal colonization levels are identical between these two strains (Fig 3.3). The toxin level at week 4 showed a trending correlation with the final tumor counts at 12 weeks by linear regression analysis (Fig 4.1C), but there was no correlation between 1-week (Fig 4.1B) or 8-week (Fig 4.1D) stool toxin levels with tumor counts, suggesting that persistent toxin A and toxin B production up to 4 weeks may be critical for tumor

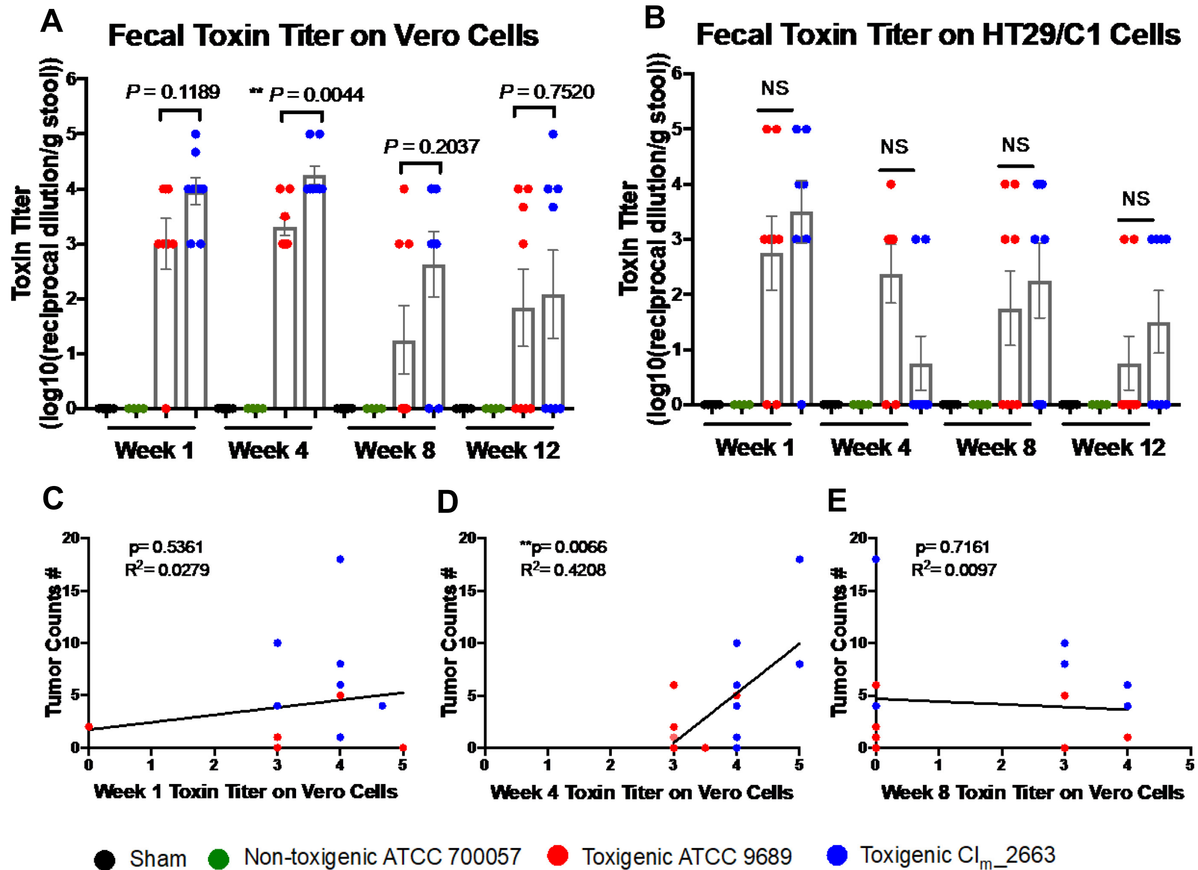


Figure 4.1 Persistent toxin production for 4 weeks correlates with colon tumor incidence. A. Fecal supernatants of mice colonized for 4 weeks with the CRC-associated strain Cl_m_2663 showed higher levels of *C. difficile* toxins in cytotoxicity assay on Vero cells. B. No differential toxin production between toxigenic strains was detected on HT29/C1 cells. C-E. Correlation between tumor counts with week 1 (C), week 4 (D), and week 8 (E) toxin titers on Vero cells. Data represent two independent mouse experiments. Each dot in the figures represents one mouse, and overlapped dots are only shown as one dot. Number of mice per group: N = 8 for each of Sham, Toxigenic ATCC9689 and Cl_m_2663 groups, and N = 4 for Non-toxigenic ATCC 700057 group. The statistical difference between two groups (A and B) was calculated with Mann-Whitney nonparametric U test. The correlation (C-E) was performed by linear regression analysis. A *P* value < 0.05 is considered significant.

Table 6. Qualitative fecal *C. difficile* toxin B ELISA		
Strains	**% of Week 1 Toxin B+ (n/N)**	**% of Week 4 Toxin B+ (n/N)**
ATCC 9689	75% (6/8)	50% (4/8)
Cl_m_2663	100% (8/8)	75% (6/8)
Chi-square P value	0.1306	0.3017
n: numbers of mice that are toxin B positive in stools; N: total mouse numbers per group		

initiation and development in *C. difficile* colonized SPF $Apc^{Min\Delta716/+}$ mice.

HT29/C1 cell cytotoxicity assays were carried out on the same fecal supernatants and did not show differential toxin titers between toxigenic ATCC 9689 strain and CRC-associated strain CI_m_2663 at any time point examined (Fig. 4.1B). Notably, we observed that 4-week fecal supernatants from mice inoculated with the CRC isolate CI_m_2663 appeared to be less cytotoxic for HT29/C1 cells as compared with Vero cells, while toxigenic ATCC strain 9689 shows similar toxin titers between Vero cells and HT29/C1 cells at each time point. This discrepancy between Vero cells vs HT29/C1 cells, together with the previous reports that Vero cell cytotoxic activity is more likely toxin B-mediated, suggested that CRC-associated strain CI_m_2663 may secrete more toxin B and similar or possibly less, toxin A compared to strain ATCC 9689. These results suggest that the toxin correlation with tumor counts might be predominantly driven by toxin B.

To further evaluate the association between toxin B and tumor formation, we tested fecal toxin B levels by ELISA. All the mice colonized with toxigenic strain ATCC 9689 or CRC-associated strain CI_m_2663 showed positive toxin B titer in 1-week stools, while 6 of 8 (75%) mice colonized with strain CI_m_2663 showed detectable fecal toxin B compared with 4 of 8 (50%) mice colonized with ATCC 9689 strain at 4 weeks after colonization (Table 6).

Given these data suggesting a potential correlation between toxin B and tumor counts, we next sought to determine the causative effect of *C. difficile* toxin B in colon

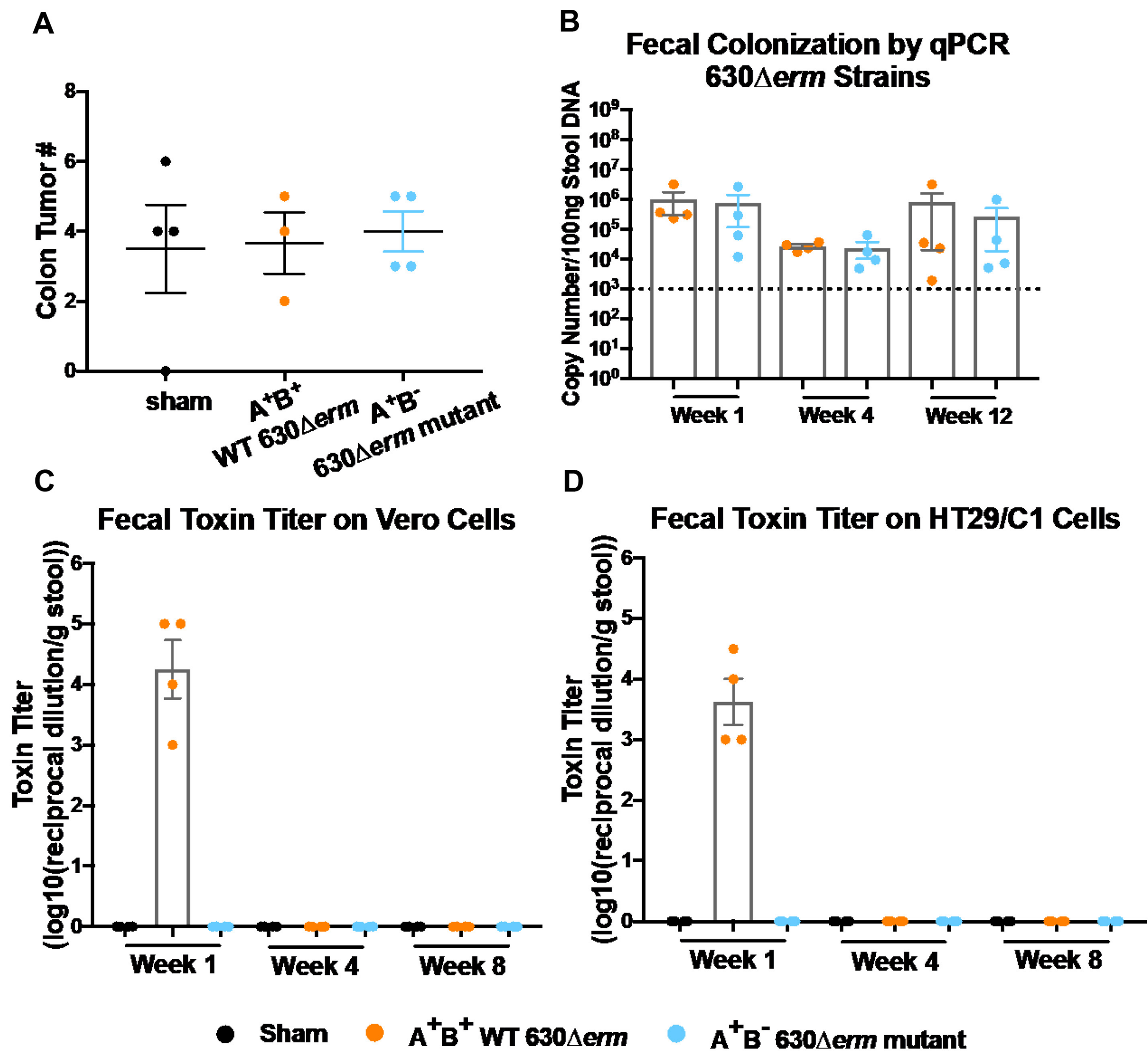


Figure. 4.2 Toxigenic *C. difficile* strain 630Δ*erm* without persistent toxin B production fails to promote tumors in *Apc*$^{Min\Delta716/+}$ mice. A. Tumor counts in mice colonized with A^+B^+ wild-type 630Δ*erm* and isogenic A^+B^- mutant as compared with sham *Apc*$^{Min/+}$ mice; B. fecal colonization assayed by qPCR using DNA isolated from Week 1, Week 4 and Week 12 stools; C-D. Toxin titer measured by cytotoxicity assays on Vero cells (C) and HT29/C1 cells (D) with fecal supernatant from mice chronically colonized with wild-type strain 630Δ*erm* and isogenic A^+B^- mutant. Data represent one single mouse experiment. The number of mice per group N = 4 except that one mouse inoculated with wild-type strain 630Δ*erm* was excluded from tumor counting (A). This mouse died one day before the harvest at 12 weeks and the colon exhibited tissue decay. But, we included the stools from all mice for the analysis of fecal colonization (B) and toxin tiers (C-D).

tumorigenesis using isogenic *C. difficile* strains, wild-type toxigenic 630Δ*erm* (*tcdA*$^+$*tcdB*$^+$) and a toxin-B-negative single mutant (A^+B^-). Here, we inoculated SPF *Apc*$^{Min\Delta 716/+}$ mice with these isogenic strains and tested tumor development. However, both wild-type 630Δ*erm* and the A^+B^- mutant strain failed to promote colon tumor formation in *Apc*$^{Min\Delta 716/+}$ mice as compared with uninfected control mice, although all the infected mice were colonized during the 12 weeks after inoculation (Figure 4.2A-B). Fecal toxin B was detected only at 1 week after colonization, but not at subsequent time points from the mice colonized with wild-type 630Δ*erm* using the Vero cell cytotoxicity assay (Figure 4.2C) and HT29/C1 cells (Figure 4.2D). Strain 630Δ*erm* has been recognized as a less virulent strain due to less toxin production and lower morbidity in murine models. However, our data suggest the level of toxin produced by strain 630Δ*erm in vivo* was similar to the other two toxigenic strains we studied at 1 week. However, the lack of persistent toxin-production after one week suggests that strain 630Δ*erm* is less virulent. In contrast with the results of Lyras *et al.* (Lyras *et al*. 2009) indicating that that the culture supernatants of the A^+B^- mutant strain induced cell-rounding mainly on HT29/C1 cells *in vitro* suggesting a toxin A effect, we found fecal supernatants from mice colonized with the A^+B^- mutant strain did not exhibit any cytotoxic activity towards either Vero cells or HT29/C1 cells at the lowest dilution (1:100) of the original stools.

4.4.2 Persistent colonization with toxigenic *C. difficile* strains induces low-grade colonic inflammation from SPF *Apc*$^{Min\Delta 716/+}$ mice after 12-week colonization. Chronic colonic inflammation has long been recognized as one of the CRC risk factors. Despite extensive data characterizing the acute inflammatory features at 1-4 days after *C. difficile*

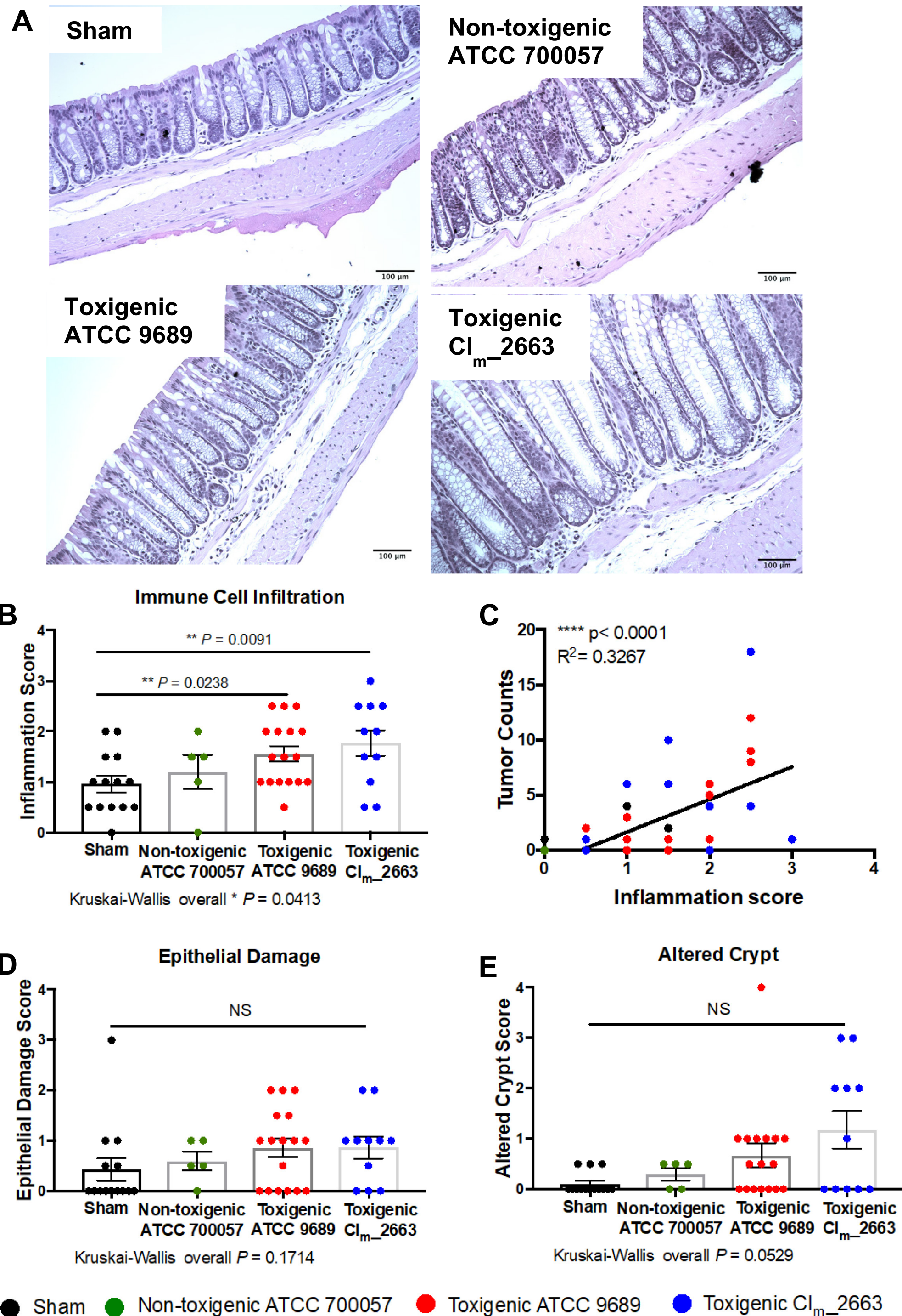
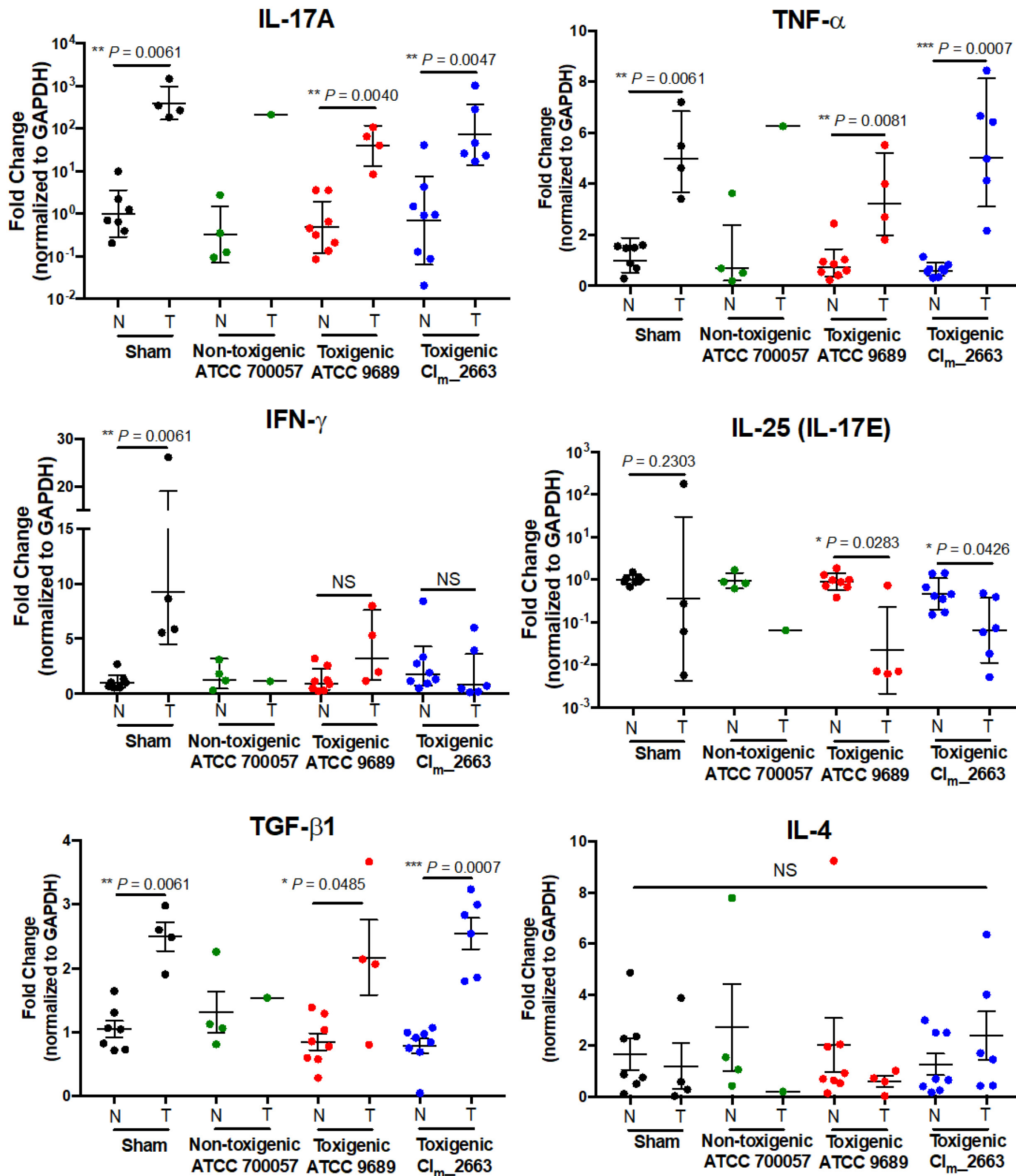


Figure 4.3 *C. difficile* chronic colonization induces low-grade chronic mucosal inflammation scattered in non-tumor colon epithelium that correlates with colon tumor formation in SPF *Apc*$^{Min\Delta716/+}$ mice at 12 weeks after inoculation. A. Mouse colon histopathology image obtained at 20x magnification, scale bar: 100 μm); B. Inflammation score; C. Correlation between inflammation score and tumor counts was performed by linear regression analysis; D. Epithelial damage score; F. Altered crypt score. Data represent at least three independent mouse experiments. The overall *P* value among 4 groups was calculated using Kruskai-Wallis test, and the statistical difference between two groups was performed using uncorrected Dunn's test. A *P* value < 0.05 is considered significant.

infection in murine models, it remains uncertain whether persistent asymptomatic *C. difficile* colonization invokes chronic inflammation that may contribute to long-term pathogenesis of chronic conditions. To evaluate *C. difficile*-induced mucosal inflammation and its correlation with colon tumorigenesis in our vancomycin and gentamicin primed SPF *Apc*$^{Min\Delta716/+}$ mouse model, we examined the colonic histopathology on H&E-stained colon sections (Figure 4.3A) at 12 weeks after *C. difficile* colonization. The overall histological evaluation suggested that the toxigenic *C. difficile* strains induced low-grade and patchy inflammation in colonic mucosa characterized by immune cell infiltration (see criteria in Table 5). The inflammatory scores show that toxigenic *C. difficile* strains, as compared with sham and non-toxigenic strain ATCC 700057, induced more infiltrating immune cells in the lamina propria (Figure 4.3B), and this inflammation correlated with colon tumor counts by regression analysis (Figure 4.3C). We did not find differential chronic epithelial damage (Figure 4.3D) or altered crypt morphology (Figure 4.3E) across the groups. These results suggest that immune mechanisms are involved in *C. difficile*-induced colon tumorigenesis.

To better understand the nature of the chronic mucosal inflammation associated with chronic *C. difficile* colonization, we analyzed cytokine expression by qPCR using colonic tissue snips from SPF *Apc*$^{Min\Delta716/+}$ mice inoculated with non-toxigenic or toxigenic strains. Generally, the cytokine gene expression identified in the normal colonic tissues was identical across the groups; however, tumors tended to show upregulation of some pro-inflammatory and regulatory cytokines. Pro-oncogenic cytokines such as IL-17A and tumor necrosis factor α (TNF-α) were highly upregulated in *Apc*$^{Min\Delta716/+}$ mouse colon



N: Normal colon tissue (no visible tumors); T: Tumors in mouse colons

● Sham ● Non-toxigenic ATCC 700057 ● Toxigenic ATCC 9689 ● Toxigenic Cl_m_2663

Figure 4.4 Colonic cytokine profile in SPF *$Apc^{Min\Delta716/+}$* mice at 12 weeks after inoculation. Inflammatory cytokine gene expression was evaluated using TaqMan gene expression assays with non-tumor normal tissues and tumors at 12 weeks after inoculation. mRNA quantities were calculated as ΔΔCT for fold-changes and normalized to mouse GAPDH. Error bars represent the mean ± SEM on a linear scale and geometric mean ± SD on a log scale. Data represent two independent mouse experiments, and each dot represents one single mouse. Statistical significance between groups was analyzed by the Mann–Whitney nonparametric U test. A $P < 0.05$ was considered statistically significant. The statistic difference between normal region and tumor tissue in Non-toxigenic ATCC 700057 group was not assessed because only 1 tumor was available. For IL-4, the overall statistics across all subgroups was performed using Kruskai-Wallis test.

tumors regardless of infection status (Fig 4.4). In contrast, IFN-γ, an anti-tumorigenic cytokine, was increased only in sham tumors but not in the tumors associated with *C. difficile* colonization, suggesting that *C. difficile* may suppress the putative IFN-γ anti-tumor immune responses. Transforming growth factor β (TGF-β), as an anti- inflammatory cytokine, was also increased in all the tumors across the infected groups and sham mice, suggesting the regulatory immune responses are at play in the tumor environment in SPF *Apc*$^{Min\Delta716/+}$ mice. IL-25, also known as IL-17E, is a cytokine that mainly promotes Th2 immune response and activates ILC2 cells. Previous studies have reported that intestinal IL-25 is suppressed during *C. difficile* infection in both humans and mouse models contributing to the pathogenesis of active colitis (Buonomo *et al*. 2016). Interestingly, IL-25 decelerates tumor cell growth *in vitro* and in xenograft mouse models (Benatar *et al*. 2010). A recent study reported that the blockade of IL-25 with neutralizing antibody increased tumor burden in a colitis-associated cancer murine model, Azoxymethane/Dextran Sodium Sulfate (AOM/DSS) model (Thelen *et al*. 2016). Here, we present that intestinal IL-25 expression was also suppressed in tumors from *Apc*$^{Min\Delta716/+}$ mice that were chronically colonized by toxigenic *C. difficile* strains. Further studies are needed to investigate whether IL-25 suppression contributes to *C. difficile*-associated tumor development, and to determine the cellular sources of IL-25 production.

We further characterized the immune cell populations infiltrating in tumors (Figure 4.5) and in non-tumor normal regions (Figure 4.6) from sham and *C. difficile*-colonized *Apc*$^{Min\Delta716/+}$ mice at 12 weeks. T cells, neutrophils and macrophages on mouse colon sections were stained using IHC antibodies against their corresponding cell surface

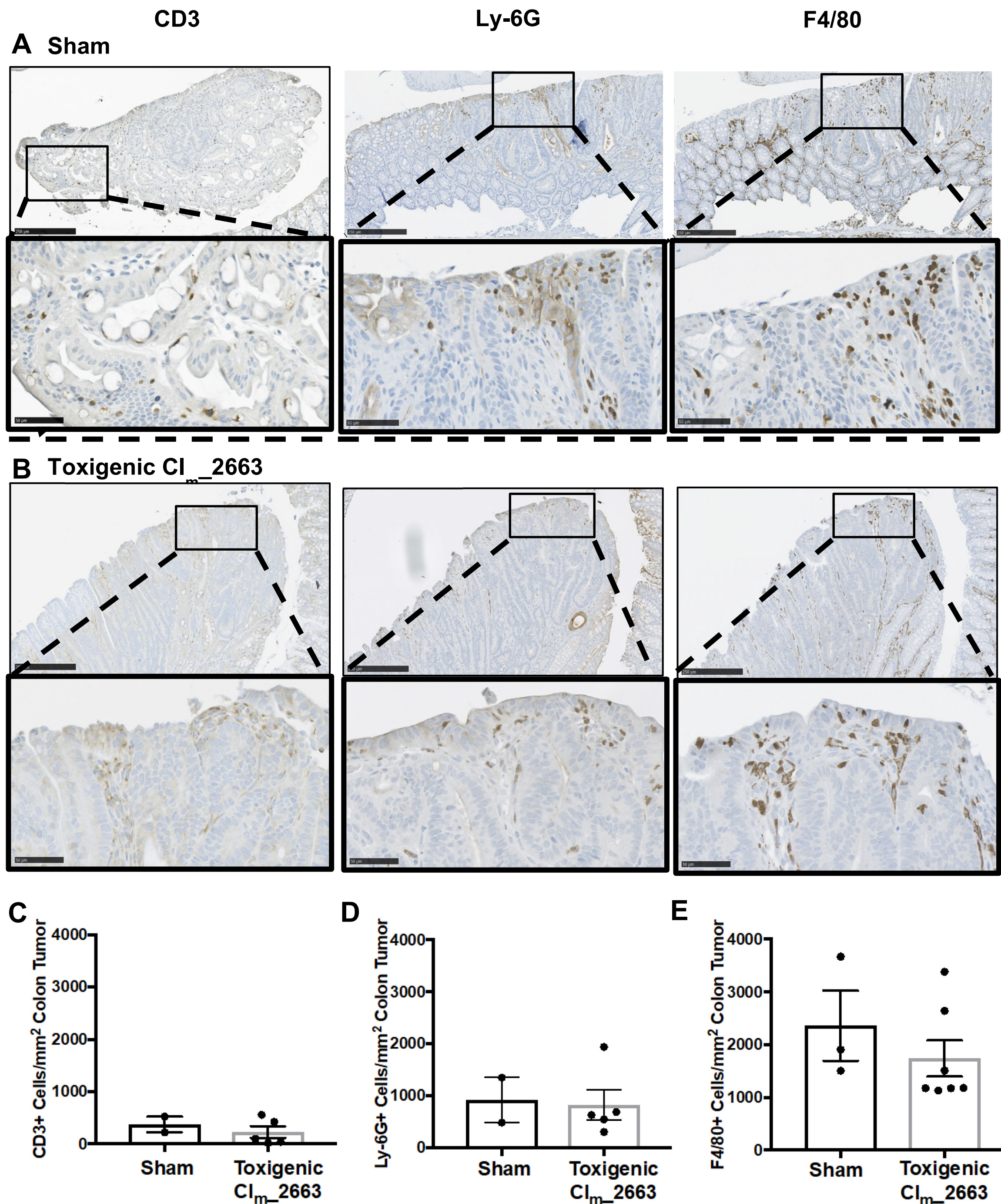


Figure 4.5 Immune cell infiltration in colon tumors from *Apc$^{Min\Delta716/+}$* mice at 12 weeks after inoculation. IHC staining of immune cells in colon tumors using antibodies against cell surface markers CD3, Ly-6G and F4/80. A. A tumor from one of the sham mice. B. A tumor from one of the CRC isolate Cl_m_2663 inoculated mice. C-E. Quantification of immune cells, CD3+ (C), Ly6G+ (D), or F4/80+ (E) cells, infiltrating in lamina propria was performed using immune cell module on Halo platform (Indica Labs). Immune cell counts were normalized by tumor area included in the analysis. Upper panel images were obtained at 10x magnification, scale bars: 250 µm. Insets were obtained at 40x magnification, scale bars: 50 µm.

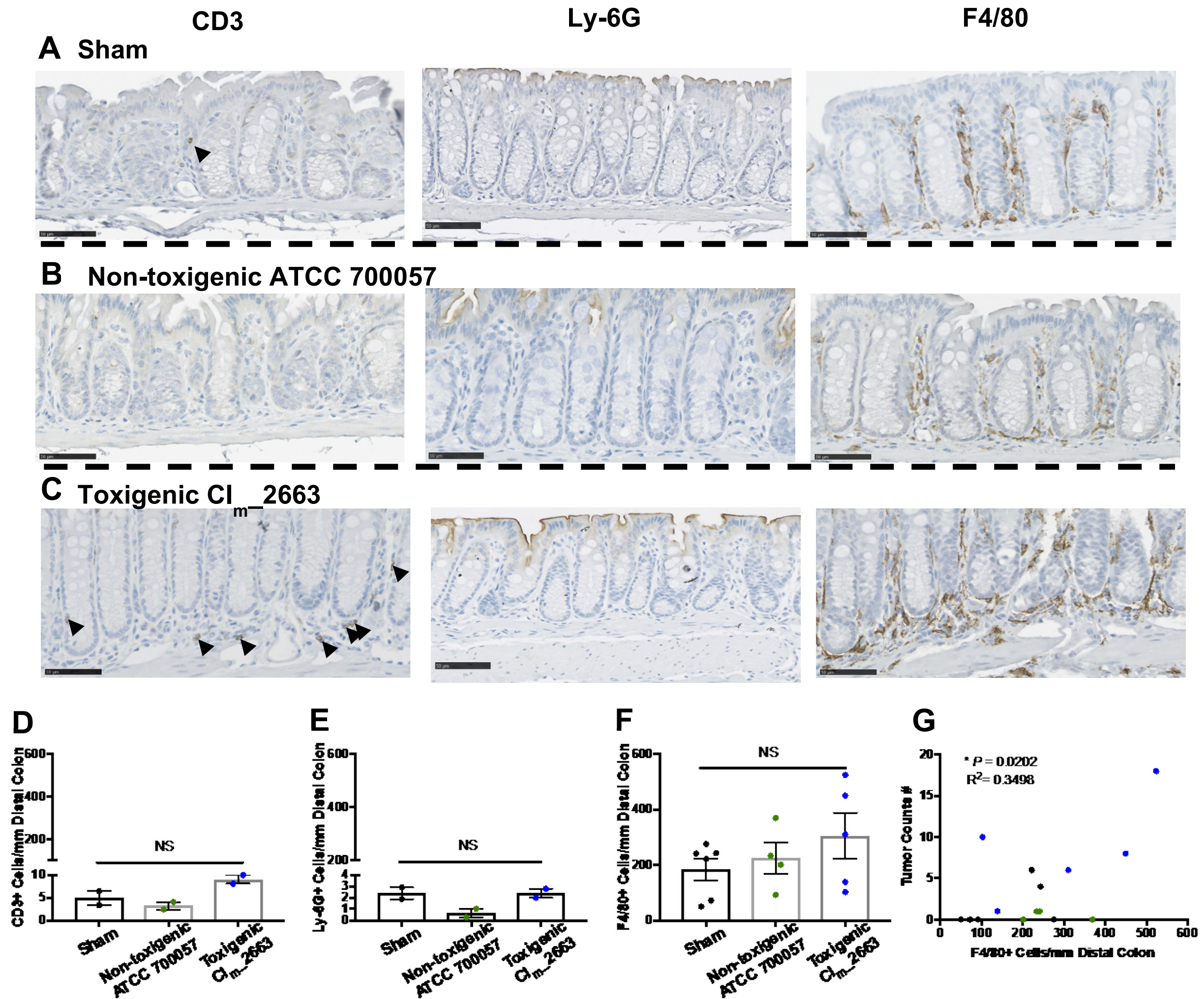


Figure 4.6 Immune cell infiltration in non-tumor normal colon regions from ***Apc***$^{Min\Delta716/+}$ **mice at 12 weeks after inoculation.** IHC staining of immune cells in colon tumors using antibodies against cell surface markers CD3, Ly6G and F4/80. A. A representative normal distal colon from one sham mouse; B. A representative normal distal colon from one mouse inoculated with non-toxigenic strain ATCC 700057; C. A representative normal distal colon from one mouse inoculated with CRC isolate Cl_m_2663. D-F. Quantification of CD3+ (D), Ly6G+ (E), or F4/80+ (F) cells in lamina propria was performed using immune cell module on Halo platform (Indica Labs). CD3+ or Ly6G+ cells were counted manually with the slide viewed on Halo. Immune cell counts were normalized by colon length included in the analysis. G. Correlation between F4/80+ macrophages and tumor counts. Images were obtained at 40x magnification, scale bars: 50 µm.

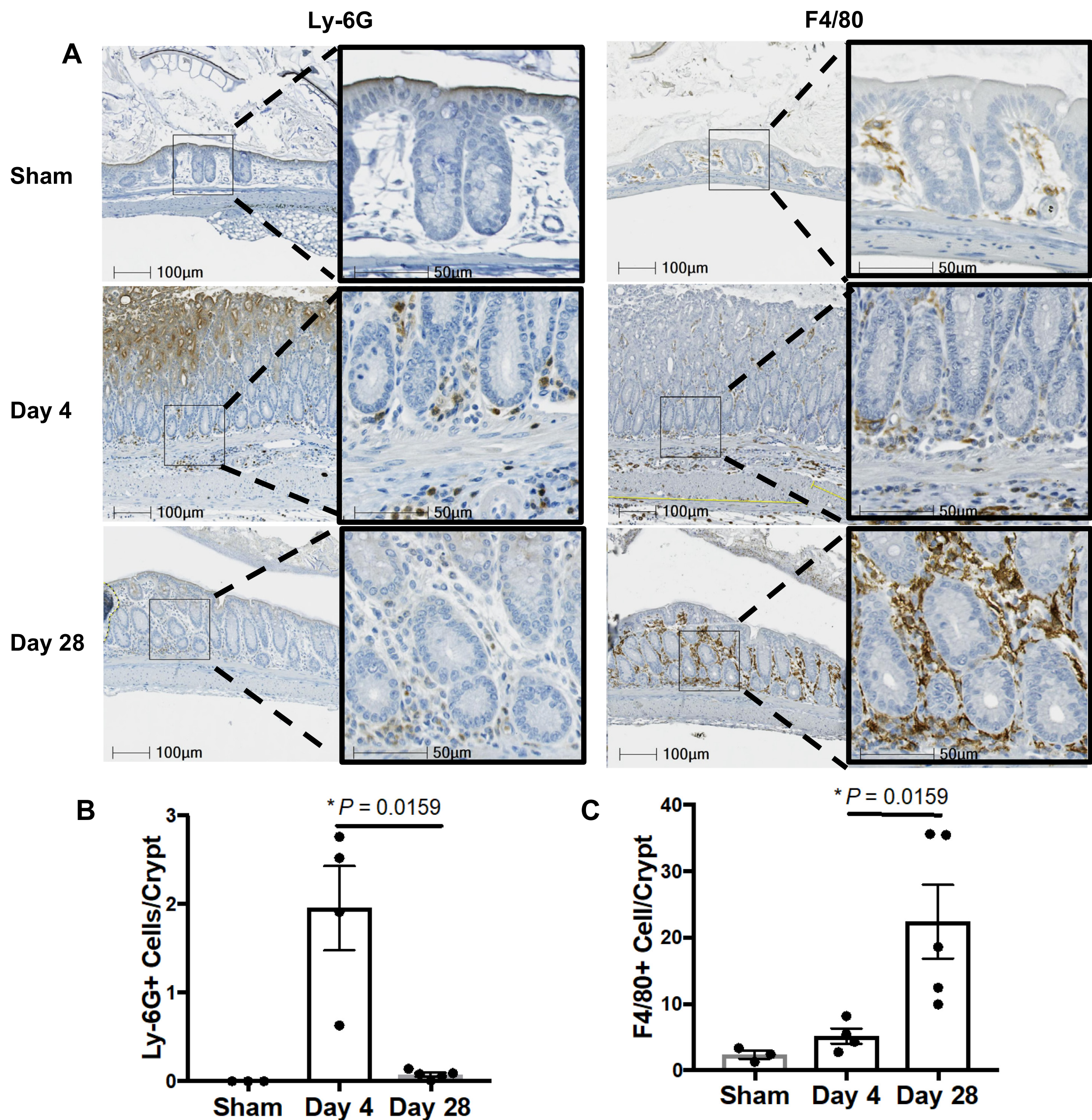


Figure 4.7 CRC Patient 3728 tumor mucosa that contains toxigenic *C. difficile* Cl$_m$_2663 induces a rapid recruitment of neutrophils but macrophages infiltrate the lamina propria over time in GF *Apc$^{Min\Delta716/+}$* mice. A. IHC staining for neutrophils or macrophages was performed using anti-Ly6G or anti-F4/80 antibodies respectively on the 3 cm distal colons from sham mice and mice inoculated with Patient 3728 tumor mucosa at Day 4 or Day 28 after inoculation. C-D. Quantification of Ly-6G+ (C) or F4/80+ (D) cells in lamina propria was performed using immune cell module on Halo platform (Indica Labs) and normalized by crypt numbers included in the analysis. Left panel images were obtained at 20x magnification, scale bars: 100 µm. Inset images were obtained at 40x magnification, scale bars: 50 µm).

markers CD3, Ly-6G and F4/80, respectively. The tumors from sham (Figure 4.5A) or CRC isolate CI_m_2663 colonized mice (Figure 4.5B) displayed similar F4/80 positive macrophages as the major infiltrating immune cell population along with mild to modest CD3 positive T cells and neutrophils at 12 weeks after inoculation. To characterize chronic inflammation induced by *C. difficile*, we examined infiltrating immune cells in non-tumor normal colon regions also at 12 weeks after inoculation. Similar to tumors, macrophages present as the major cell type infiltrating into the lamina propria across the groups (Figure 4.6), whereas some patchy T cell infiltration and rare neutrophils were found by IHC straining. Macrophage infiltration does not appear to depend on *C. difficile* infection but is trending to correlate with tumor burden. Together, our results show that the chronic colonization with CRC isolate CI_m_2663 may only provoke low-grade inflammation that is unlikely to distinguish from sham mice or mice inoculated with non-toxigenic strain ATCC 700057 at 12 weeks after inoculation. Macrophages in the colon mucosa of *Apc*$^{Min\Delta716/+}$ mice (Fig 4.6) are predominant cell population along with the tumorigenic process.

To further evaluate whether the macrophage recruitment marks *C. difficile*-induced chronic inflammation, we assessed the transition of macrophage infiltration in the colon mucosa from day 4 to day 28 in GF *Apc*$^{Min\Delta716/+}$ mice colonized with human CRC tumor mucosal homogenate from Patient 3728. Ly-6G+ neutrophils showed a rapid and temporary response to the homogenate inoculum that includes *C. difficile*. The F4/80 IHC staining on colon sections demonstrated that macrophages are increasingly recruited into the colon lamina propria at day 28 relative to day 4 after inoculation with Patient 3728 tumor mucosal homogenate when compared to sham GF *Apc*$^{Min\Delta716/+}$ mice. Our whole

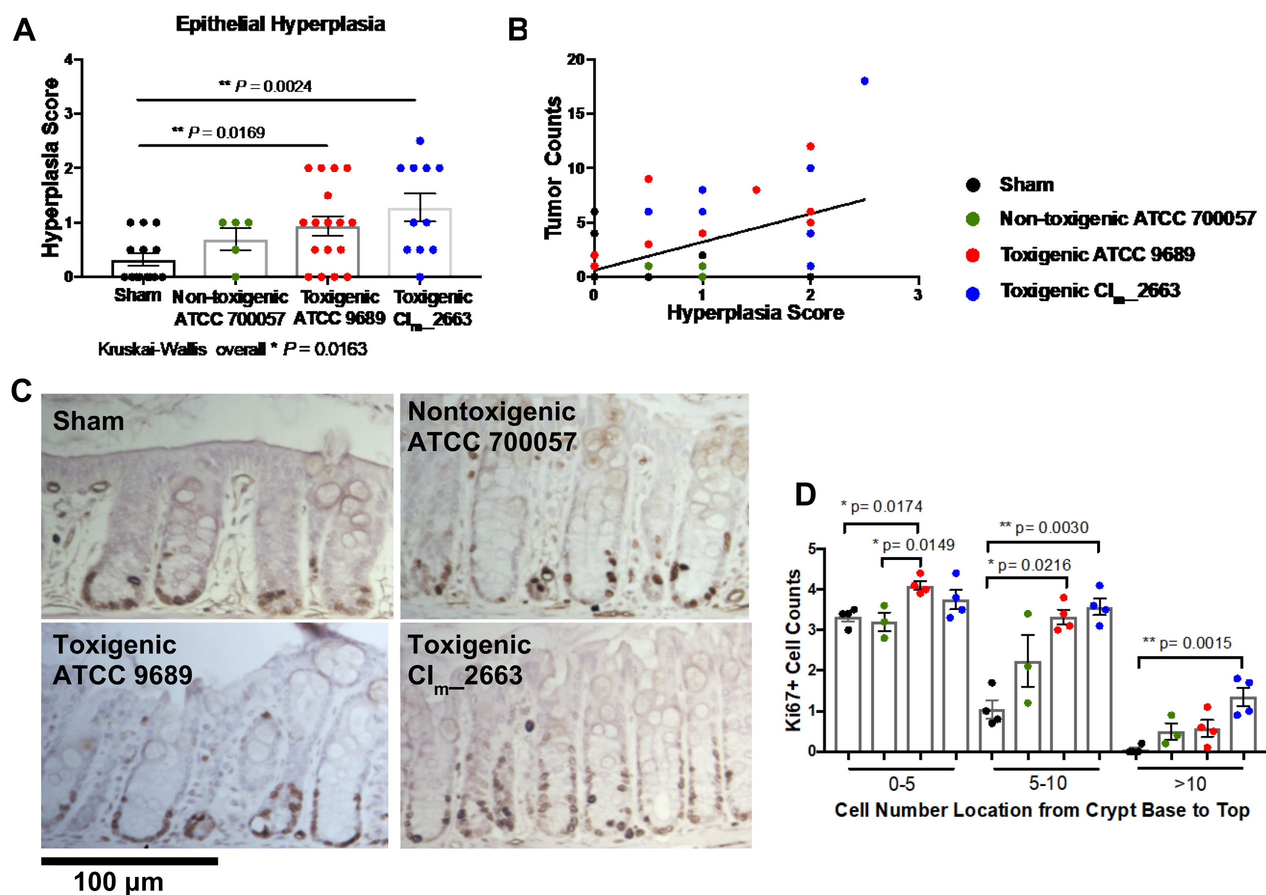


Figure 4.8 Low-grade chronic inflammation in non-tumor colon tissues is accompanied by epithelial cell proliferation at 2 weeks and 12 weeks after colonization. A. Hyperplasia score evaluated based on crypt length on H&E-stained colon sections at 12 weeks after inoculation; B. Hyperplasia score at 12 weeks correlates with tumor counts. Data in A and B represent at least three independent mouse experiments, and each dot represents one single mouse; C. IHC-stained epithelial cells using anti-Ki-67 antibody on non-tumor normal regions of distal colon sections at 2 weeks after *C. difficile* inoculation; D. Quantification of Ki-67+ cells along the colonic crypt axis from base to top. 4 or 5 locations were randomly selected for quantification of Ki-67+ cells. Each half of 2 well-oriented crypts on one photograph was selected for total cell and Ki-67+ cell counting. Results were quantified from the crypt bottom (0-5 cells from the crypt bottom) to the crypt top (>10 cells from the crypt bottom). The overall *P* value among 4 groups was calculated using Kruskai-Wallis test, and the statistical difference between two groups was performed using uncorrected Dunn's test. A *P* value < 0.05 is considered significant.

genome sequencing data showed that *C. difficile* isolate #3728-3 from the tumor mucosal sample of Patient 3728 is genetically identical to our isolate CI_m_2663 isolate from GF mice inoculated with same patient sample (Figure 3.9). CD3+ T cells were rarely detected by IHC staining (data not shown).

4.4.3 Promotion of epithelial proliferation may be pro-tumorigenic when *Apc*$^{Min\Delta716/+}$ mice are colonized chronically with toxigenic *C. difficile* strains. We aimed to address whether epithelial cell biology was changed when *Apc*$^{Min\Delta716/+}$ mice were colonized mouse colon tumor counts (Figure 4.8B). In contrast, mice inoculated with non-toxigenic *C. difficile* strain showed similar histopathology to sham mice. To evaluate whether low-grade mucosal inflammation induced by toxigenic *C. difficile* strains is accompanied by epithelial cell proliferation, we examined cell proliferation by IHC using an antibody against the nuclear protein Ki-67 (Figure 4.8C) at 2 weeks after *C. difficile* colonization in SPF *Apc*$^{Min\Delta716/+}$ mice. Similar to the colon epithelial hyperplasia at 12 weeks, Ki-67 positive cells were significantly increased in mice colonized with toxigenic *C. difficile* strains at 2 weeks after colonization (Figure 4.8C). This result suggests that toxigenic *C. difficile* strains (and hence their secreted toxins) associate with both early and long-lasting colon epithelial cell proliferation, thereby possibly facilitating tumor development.

To determine whether pro-tumorigenic signaling pathways in colonic epithelial cells are altered in the context of toxigenic *C. difficile* and chronic inflammation, we quantified Wnt/β-catenin regulated genes including c-Myc, Lgr5, Axin2, CCND1 and CD44 (Table 5)

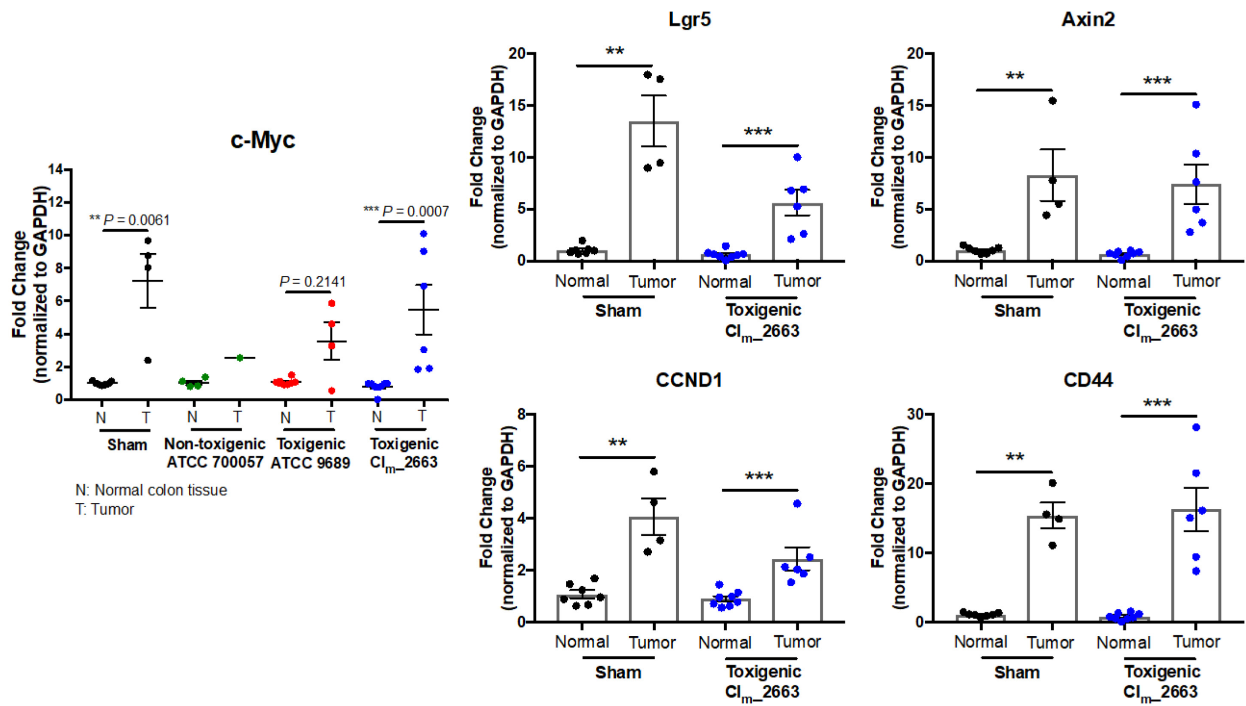


Figure 4.9 The expression of Wnt/β-catenin regulated genes in normal colonic tissues and tumors in SPF *Apc*$^{Min\Delta716/+}$ mice at 12 weeks after inoculation. Wnt/β-catenin downstream genes that were examined in this study include c-Myc, Lgr5, Axin2, CCND1 and CD44 in normal colons relative to tumors using RT-qPCR assay. Data represent two independent mouse experiments, and each dot represents one single mouse. Statistical significance between groups was analyzed by the Mann–Whitney nonparametric U test. A $P < 0.05$ was considered statistically significant. ** $P < 0.01$, *** $P < 0.001$.

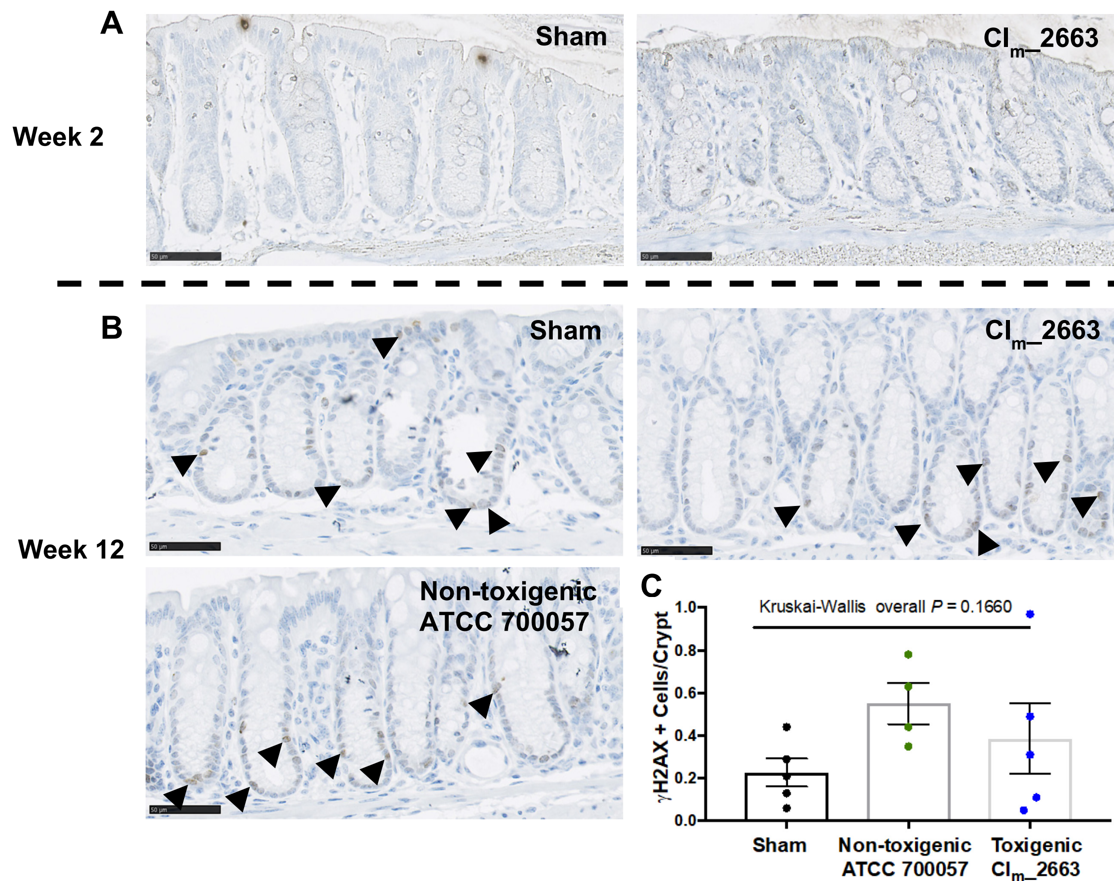


Figure 4.10 DNA damage assessment by γ-H_2AX staining in SPF *Apc*$^{Min/+}$ mice at 2 weeks and 12 weeks after inoculation. The epithelial cells with more than four γ-H_2AX+ nuclear foci (r-foci) were counted as rH_2AX+ cells. A. IHC-stained mouse distal colon for γ-H_2AX at 2 weeks after inoculation with Cl_m_2663 as compared with sham mice. Data at 2 weeks were not quantified as very few γ-H_2AX+ cells/condition were identified. Images present one mouse experiment with 4 mice/group; B. γ-H_2AX staining on colon sections at 12 weeks and treatment conditions include Sham, Non-toxigenic ATCC 700057 and Cl_m_2663. Black arrow heads identify γ-H_2AX+ epithelial cells. C. γ-H_2AX+ epithelial cells/crypt were quantified at 12 weeks. Images present two separate mouse experiments with 4-5 mice/group. Images were obtained at 40x magnification, scale bars: 50 μm.

in normal colon tissues relative to tumors using RT-qPCR assays. All these genes were upregulated in *Apc*$^{Min\Delta716/+}$ mouse colon tumors suggesting that Wnt signaling in tumors is activated independent of the status of *C. difficile* infection and toxin production (Figure 4.9), a result consistent with use of the *Apc*$^{Min\Delta716/+}$ mouse model. However, the increased expression of these genes was not detected in non-tumor colon tissues, indicating that Wnt/β-catenin signaling in normal colon was not altered by toxigenic *C. difficile* colonization, at least, at the time point examined.

Next, we evaluated DNA damage using an IHC antibody against phosphorylated histone H_2AX (γ-H_2AX is the most abundant phosphoepitope and used as the marker of DNA damage) on mouse colon sections at multiple time points. The results showed that toxigenic *C. difficile* colonization did not augment DNA damage in normal colon tissues either at 2 weeks or at 12 weeks after inoculation, although we observed γ-H_2AX+ cells more frequently at 12 weeks than 2 weeks.

4.5 Discussion

As described to date, CDI is a toxin-mediated colonic disease. However, humans infected by the same epidemic strain often show a wide range of clinical outcomes from asymptomatic colonization, mild diarrhea through more severe and even life-threatening disease such as pseudomembranous colitis. We herein hypothesize that toxin production by *C. difficile in vivo* may be regulated differentially between individuals leading to variable host immune responses and disease courses. For the first time to our knowledge, we evaluated longitudinal *in vivo* toxin production in a mouse model. Although *C. difficile* toxin

induces acute colitis, very little is known about the time course and/or role of persistent *C. difficile* toxin production in fostering chronic colon disease. Our results suggest that persistent toxin production for 4 weeks correlated with the final tumor counts at 12 weeks. This result is further supported by the fact that toxigenic *C. difficile* strain 630Δ*erm* that produced toxins only for one week *in vivo* failed to enhance tumorigenesis (Figure 4.2). Although this correlation is insufficient to firmly identify a role for the *C. difficile* toxins in tumorigenesis, our results highlight the merit of measuring toxin production longitudinally in chronic disease evaluation. The combination of toxin assessment over time in humans who are asymptomatic *C. difficile* carriers and corresponding colonic immune responses may help to inform the pathogenesis of chronic *C. difficile* colonization and its potential impact on chronic colon disease(s).

In order to determine the role of toxin B in colon tumor formation, we utilized wild-type 630Δ*erm* and isogenic A^+B^- mutant *C. difficile* strains generated by Dr. Dena Lyras (Lyras *et al*. 2009) in our mouse model. In contrast to previously published studies, data from our mouse model drew our attention to some unique aspects. First, in the pilot study with a limited number of mice (Figure 4.2A), wild-.type 630Δ*erm* did not enhance tumor incidence after 12 weeks of colonization in $Apc^{Min\Delta716/+}$ mice, indicating that the *C. difficile* pro-tumorigenic capability may be a strain-specific property. Second, previous studies reported that 630Δ*erm* is a less virulent strain due to the lower toxin production *in vitro*. However, we found similar levels of cytotoxicity with one-week fecal samples in $Apc^{Min\Delta716/+}$ mice colonized with 630Δ*erm* and $Apc^{Min\Delta716/+}$ mice colonized with two other toxigenic *C. difficile* strains (compare Fig 4.1 and 4.2). The most obvious difference

between the pro-tumorigenic *C. difficile* strains (CI_m_2663 and ATCC 9689) and 630Δ*erm* is that 630Δ*erm* strain lacked the capability to sustain toxin production beyond one week, suggesting that persistent toxin-production may be critical to the ability of a *C. difficile* strain to induce colon tumorigenesis and possibly other colon chronic diseases. Alternatively, there may be additional differences such as mucosal adhesion or toxin delivery mechanisms that contribute to the inability of the 630Δ*erm* *C. difficile* strain to induce colon tumorigenesis. Although in the original Lyras' study (Lyras *et al*. 2009), the 630Δ*erm* isogenic A^+B^- mutant strain was mostly avirulent in a hamster model, they only tested the presence of toxin A and the absence of toxin B using an *in vitro* cytotoxicity assay with *C. difficile* culture broth. Toxin A and toxin B production *in vivo* along with disease status was not assayed. *C. difficile* toxin production has been suggested to be a process highly regulated by the local environment, i.e., the colon. Our work emphasizes the importance of assessing *in vivo* toxin production over time in defining the pathogenesis of *C. difficile* infection in animal models. Consistent with other studies, Vero cells and HT29/C1 cells are both susceptible to toxin A and toxin B. Discerning the cytopathic effect of toxin B from toxin A in mouse stools using these two cell lines is insecure, however our results (Figure 4.1A-B) tentatively represent differential cytotoxic effects of toxin A or toxin B. Isogenic mutants of A^+B^-, A^-B^+ and A^-B^- strains are required to definitively answer these questions.

We established a mouse model supporting *C. difficile* persistent colonization and demonstrated the unique features of chronic mucosal inflammation by histologic evaluation, colonic cytokine expression and immune cell infiltration after 12 weeks of

colonization. We observed that mice colonized with toxigenic *C. difficile* strains tend to show a correlation between higher scores of inflammation and hyperplasia and tumorigenesis in SPF *Apc*$^{Min\Delta716/+}$ mice. Although toxigenic *C. difficile* strains induce significant upregulation of colonic cytokine expression, particularly TNF-α, NOS-2, IL-17A, in GF wild-type mice after 2-week colonization, these cytokines in infected SPF *Apc*$^{Min\Delta716/+}$ mice remain similar to sham at 2 weeks or 12 weeks in non-tumor normal colon tissues. Our findings of an association between inflammation (Figure 4.3) and hyperplasia (Figure 4.8) with tumor multiplicity suggest that low-grade mucosal inflammation is associated with tumorigenesis in our antibiotic-treated SPF *Apc*$^{Min\Delta716/+}$ mouse model. However, additional studies are needed to define the tumor-promoting cells and/or molecules such as cytokines involved in *C. difficile*-induced tumorigenesis.

Our findings present a differential inflammation-dependent mechanism involved in *C. difficile*-induced tumorigenesis as compared with enterotoxigenic *Bacteroides fragilis* (ETBF)-induced tumors in SPF *Apc*$^{Min\Delta716/+}$ mice where IL-17A-associated inflammation is a hallmark of the tumorigenic microenvironment (Wu, S. *et al*. 2009; Chung *et al*. 2018; Thiele Orberg *et al*. 2017; Hurtado *et al*. 2018). Our preliminary investigations to characterize *C. difficile*-induced low-grade chronic inflammation in distal colons by immunohistochemistry suggested that predominant F4/80+ macrophages infiltrating into lamina propria and tumors may contribute to tumorigenic environment induced by toxigenic *C. difficile* strains. Neutrophils appear to be a fast but transient responder to *C. difficile* infection. As less abundant on colon sections by IHC, CD3+ T cells from lamina propria may need to be enriched and profiled in future studies by flow cytometry to testify

the role of IL-17A-associated inflammation in *C. difficile*-induced tumorigenesis. To characterize early inflammation after *C. difficile* colonization in *Apc*$^{Min\Delta716/+}$ mice will inform the inflammatory signatures better than the low-grade chronic inflammation. More importantly, acute inflammation likely diminishes epithelial barrier function (increasing colon permeability) that is considered a key feature of early colon tumorigenesis and likely persists as the chronic inflammatory response develops (Grivennikov *et al*. 2012).

Additionally, histologically scored epithelial hyperplasia at 12 weeks and quantified Ki-67-marked cell proliferation at 2 weeks are consistent with the hypothesis that the low-grade chronic inflammation may foster a persistent proliferative, pro-tumorigenic mucosal environment. This is consistent with previous work by our laboratory that shows that human colonic biofilms predispose normal colon tissue to a pro-carcinogenic status with epithelial hyperproliferation (Dejea *et al*. 2014). We also tested whether the pro-tumorigenic status of the colonic epithelial cells involves Wnt/β-catenin signaling activation or DNA damage. Our results showed that Wnt/β-catenin target gene expression and colon epithelial cell DNA damage are enhanced in tumors but not detected in normal colon tissues of both sham and toxigenic *C. difficile* chronically colonized SPF *Apc*$^{Min\Delta716/+}$ mice at 12 weeks. Thus, differential Wnt signaling and/or colon epithelial cell signaling was not seen with *C. difficile* colonization at the time points examined. Future work on the time course and mechanisms of colon epithelial cell signaling, DNA damage and immune responses in the colon in response to chronic *C. difficile* colonization is warranted.

4.6 Conclusion

Our results show that persistent production of *C. difficile* toxins in the genetically susceptible (i.e., *Apc*$^{Min\Delta716/+}$) SPF mouse colons is associated with excess colon tumor development. A critical question is to precisely determine the contributions of *C. difficile* toxin A and/or toxin B in colon tumor promotion by *C. difficile*. To accomplish this goal, the isogenic mutants of $A^{+}B^{-}$, $A^{-}B^{+}$ and $A^{-}B^{-}$ strains on the genetic background of CRC CI_m_2663 will need to be constructed and tested in our chronic colonization mouse model. Our results also suggest that the unique mechanism(s) by which, at least, certain toxigenic *C. difficile* strains promote tumorigenesis likely involves elements of acute then chronic inflammation. Macrophages are predominant cell population in the colon mucosa of *Apc*$^{Min\Delta716/+}$ mice accumulating over time along with the tumorigenic process. In future studies, we propose that detailed immunologic profiling and RNA-Seq, particularly of the myeloid and T cell populations, may yield insights into a pro-tumorigenic colon mucosal environment that fosters persistent colon epithelial hyperplasia and dysplasia.

Chapter 5

Summary

5.1 General discussion. Increasing studies in recent years highlight the important feature that polymicrobial biofilms are associated with or contribute to the pro-tumorigenic microenvironment in which human CRC arises. A few studies (Wong *et al*. 2017; Li *et al*. 2019; Tomkovich *et al*. 2019) have revealed that mucosal or fecal microbiota from CRC patients induce tumor formation in murine models. Thus, the identification of pro-carcinogenic driver bacterial species or microbial consortia is an urgent and necessary question in this field. The mechanisms by which biofilms promote tumor development can range from microbial virulence to host immune activation and altered epithelial biology. 16S rRNA amplicon profiling in the studies presented in this thesis identified *C. difficile* as a consistent species present in human BF+ colonic mucosa that subsequently colonized mouse mucosa and feces in mice inoculated with human BF+ mucosal homogenates. However, whether and how *C. difficile* may contribute to CRC remains unknown. We aimed to test the tumorigenic potential and the capability to assemble biofilms of *C. difficile* using the genetic murine model of CRC, *Apc*$^{Min/+}$ mice, known to be susceptible to intestinal tumor formation. We characterized the unique features associated with *C. difficile*-induced tumors and elucidated some of the mechanisms by which *C. difficile* may mediate colon tumorigenesis. Our results will inform future human epidemiologic studies to identify the relationship between *C. difficile* carriage and the risk of CRC development.

In this study, we established a mouse model to foster *C. difficile* chronic colonization and

inoculation of SPF *Apc*$^{Min\Delta716/+}$ mice with toxigenic *C. difficile* strains, particularly our CRC-associated isolate, yielded a colon tumorigenic phenotype with colon tumor multiplicity. After strain inoculation and mouse colonization, *C. difficile* strains (non-toxigenic ATCC 700057, toxigenic ATCC 9689 and toxigenic CI_m_2663), regardless of carrying toxin A and toxin B, displayed largely similar levels of fecal bacterial burden at each of the time points evaluated during 12-week experiments. However toxigenic strains (*tcdA*$^+$ *tcdB*$^+$) significantly enhanced tumor incidence as compared with the non-toxigenic strain (*tcdA*$^-$ *tcdB*$^-$). These findings suggested that *C. difficile* promotes colon tumorigenesis that is dependent on a toxin A and toxin B production. One limitation of these experiments is the non-toxigenic strain ATCC 700057 is not an isogenic mutant generated from either of the toxigenic strains (ATCC 9689 and CI_m_2663). Hence, we cannot exclude the tumorigenic potential of other *C. difficile* virulence factors that differ genetically in these strains. A thorough genomic comparison of these three strains may provide important clues to the identification of new virulence factors and development of specific toxin-knockout strains from *C. difficile* strain CI_m_2663 will further test the specific importance of Toxin A and/or Toxin B in *C. difficile*-induced colon tumorigenesis (in progress).

C. difficile 16S rRNA-specific qPCR analysis of normal colonic tissues and tumors found that *C. difficile* did not selectively accumulate in tumor areas of *Apc*$^{Min\Delta716/+}$ mice. FISH analysis with a *C. difficile* 16S rRNA specific probe demonstrated the vast majority of *C. difficile* cells localized in the colon lumen with sparse invasion into the inner mucus layer in the colons of both GF wild-type mice and SPF *Apc*$^{Min\Delta716/+}$ mice. Our results clearly showed that *C. difficile* strains, including the isolate from CRC-associated biofilms, did

not re-assemble mucus-invasive biofilms that adhere to colonic epithelium after 2 weeks of colonization. These findings could suggest that *C. difficile* serves as a driver bacterium in colon tumorigenesis and/or works with a bacterial consortium to modulate epithelial biology. These possibilities encourage us to further consider the role of secreted molecules, particularly toxins, as well as putative *C. difficile*-associated quorum-sensing mechanisms as possibilities for direct (e.g., toxin) and/or indirect (e.g., inflammation) pro-tumorigenic *C. difficile*-epithelial cell interactions.

Given the importance of *C. difficile* toxins in colitis pathogenesis (Chandrasekaran and Lacy 2017; Viswanathan *et al*. 2010; Kuehne *et al*. 2014) and controversial effects toxin B on tumorigenic signaling (Na *et al*. 2005; Tao *et al*. 2016; Chen, P. *et al*. 2018), *C. difficile* toxins, particularly toxin B, are likely to modulate the tumor microenvironment. We investigated whether toxin A and toxin B production may, at least partly, contribute to the tumor multiplicity *in vivo*. To quantify toxin A and toxin B production, we performed cytotoxicity assays on Vero cells and detected higher cytotoxicity titers with the fecal samples from the mice colonized with the CRC-associated isolate. This strain appeared to induce more tumors in $Apc^{Min\Delta716/+}$ mice. In contrast, toxin A and toxin B production was not detected in the feces of the mice colonized with a different toxigenic *C. difficile* strain, wild-type 630Δ*erm* ($tcdA^{+}$ $tcdB^{+}$), at the 4-week time point. This strain also did not promote colon tumor formation in $Apc^{Min\Delta716/+}$ mice. Together these data suggested an important correlation between the persistent production of *C. difficile* toxins and colon tumorigenesis in our CRC murine model. However, it remains unclear how toxin A and toxin B production is regulated *in vivo* and how this persistent toxin stress impacts colon epithelial cell and

mucosal immune cell functions to contribute to colon tumor initiation and/or subsequent tumor progression. Nevertheless, our results highlight the need to investigate longitudinal *C. difficile* toxin production in human patients with CDI or asymptomatic carriers to determine if a subset of CDI patients develop chronic colonization with persistent low level mucosal inflammation putatively conducive to the development of CRC over time. This may be particularly relevant in patients with IBD where both *C. difficile* persistence in the colon and an increased risk for colitis-associated CRC is known (D'Aoust *et al*. 2017; Baker *et al*. 2019).

To demonstrate the features of chronic mucosal inflammation caused by 12-week *C. difficile* colonization, we evaluated mouse colon histology and mucosal immune cell infiltration using immunohistochemistry. The inflammation score and hyperplasia score were increased by toxigenic *C. difficile* strains and correlated with tumorigenesis in SPF $Apc^{Min\Delta716/+}$ mice. Of interest, the toxigenic *C. difficile* strains induced significant colonic cytokine expression, particularly TNF-α, NOS-2, IL-17A and IL-22, in the colon epithelium of GF wild-type mice after 2-week colonization. However, we did not detect differential up-regulation of these cytokines in infected SPF $Apc^{Min\Delta716/+}$ mice at 2 weeks or 12 weeks, suggesting a low-grade, subclinical mucosal inflammation may contribute to tumorigenesis in our antibiotic-treated SPF mouse model. These findings present a different mechanism when compared to ETBF-induced tumorigenesis in SPF $Apc^{Min\Delta716/+}$ mice in which IL-17A-associated inflammation is a hallmark of the tumorigenic microenvironment (Wu, S. *et al*. 2009; Chung *et al*. 2018). Our preliminary investigations to characterize *C. difficile*-induced low-grade chronic inflammation in distal colons by

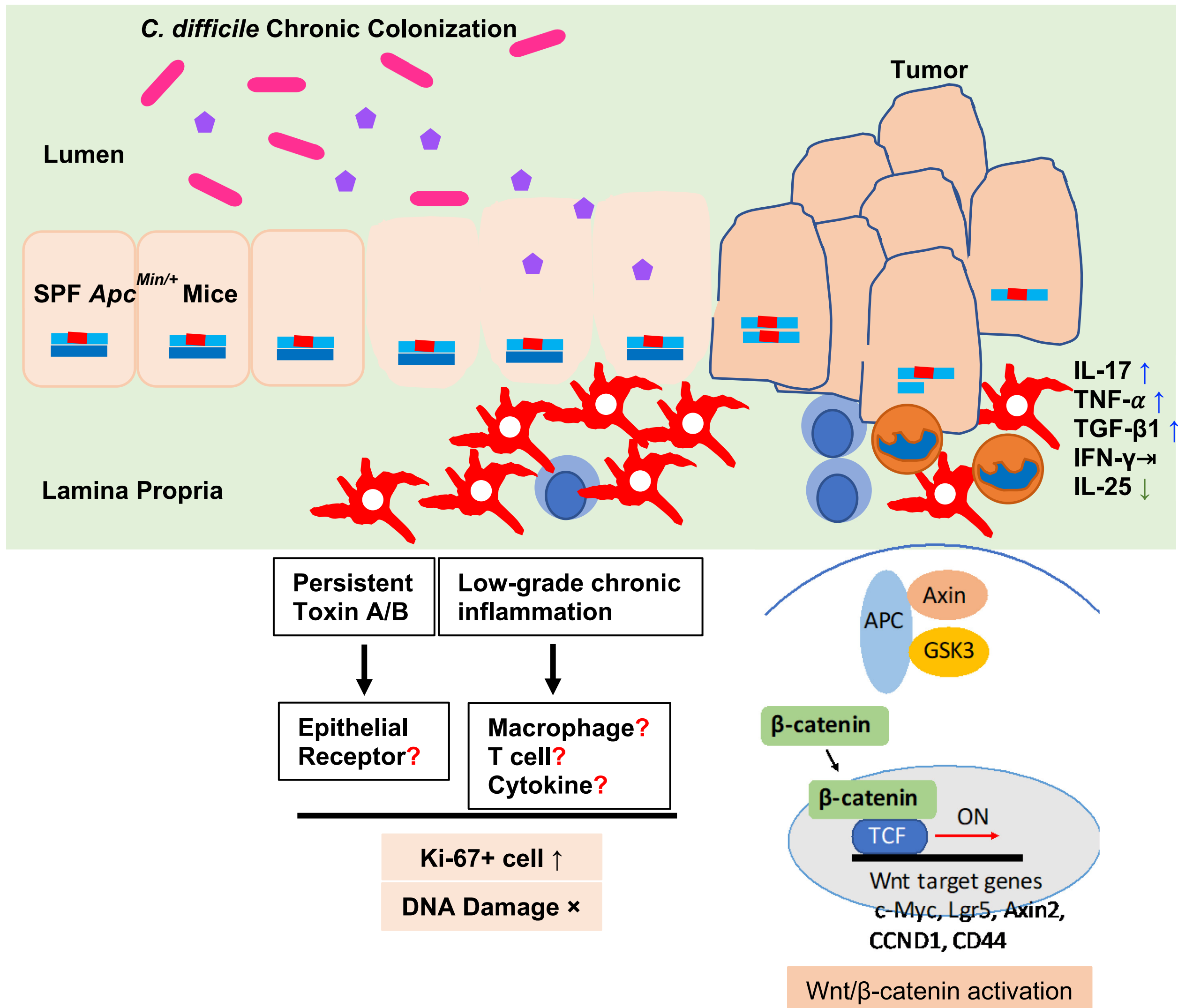


Figure 5.1 Schematic diagram of the mechanisms by which *C. difficile* enhances colon tumorigenesis in *Apc*$^{Min\Delta 716/+}$mice.

immunohistochemistry suggested that predominant F4/80+ macrophage and possible CD3+ T cells infiltrating into lamina propria may contribute to tumor development induced by toxigenic *C. difficile* strains. This low-grade chronic inflammation is accompanied by histologically-scored epithelial hyperplasia at 12 weeks and quantified Ki-67-marked cell proliferation at 2 weeks, suggesting a pro-tumorigenic, proliferative mucosal state. However, Wnt/β-catenin target gene expression and epithelial DNA damage are enhanced in tumors but not detected in normal colon tissues at 12 weeks and/or 2 weeks after *C.*

difficile colonization in SPF $Apc^{Min\Delta716/+}$ mice, hereby suggesting pro-tumorigenic signaling in epithelial cells is not differentially accumulating with *C. difficile* chronic colonization. The chronic inflammation induced by the *C. difficile* persistence in both humans and mice is an open field for further research and may contribute to the clinical risk evaluation of *C. difficile* asymptomatic or symptomatic carriers in the long term.

5.2 Conclusion

In this project, we demonstrate that chronic colonization of toxigenic *C. difficile* strains, particularly our CRC-associated isolate CI_m_2663, but not the non-toxigenic strain, induce colon tumorigenesis in $Apc^{Min\Delta716/+}$ mice without forming a biofilm structure. Persistent *C. difficile* toxin A and toxin B production and mucosal inflammation correlate with colon tumor multiplicity. A chronic inflammatory response, likely provoked by *C. difficile* toxin A and/or toxin B, is marked by accumulating macrophage infiltration and crypt hyperplasia, both considered to play a role in pro-carcinogenesis. Collectively, Figure 5.1 displays our current model for potential mechanisms by which *C. difficile* induces colon tumorigenesis.

5.3 Future directions

Prior investigations (Martin-Verstraete *et al*. 2016; Darkoh *et al*. 2015) have shown that *C. difficile* toxin production is largely regulated by the environment such as by nutrients, antibiotic treatment, temperature and pH. In this study, we present data showing a clear tumorigenic phenotype driven by toxigenic *C. difficile* strains that persistently produce toxin A and toxin B *in vivo*. Therefore, one of the future directions is to compare the tumor multiplicity and longitudinal toxin production in the feces from mice colonized with different

C. *difficile* strains under different environmental conditions such as diverse antibiotic regimens. An important next step is to discern the contribution of toxin A and toxin B in tumorigenesis by generating isogenic strains including *tcdA*$^{+}$ *tcdB*$^{-}$, *tcdA*$^{-}$ *tcdB*$^{+}$, and *tcdA*$^{-}$ *tcdB*$^{-}$ as compared with the parental CRC-associated isolate CI_{m}_2663. As noted, the isolation and evaluation of immune cell populations from mouse colonic mucosa by high parameter flow cytometry and/or RNA-Seq analyses will enable us to further understand the specific immune cell biological activities induced by differing *C*. *difficile* strains, the contribution of toxin A and/or toxin B to mucosal inflammation in *Apc*$^{Min\Delta716/+}$ mice and, ultimately, the impact on colon tumorigenesis.

Reference

Abt, M.C., B.B. Lewis, S. Caballero, H. Xiong, R.A. Carter, B. Susac, L. Ling, I. Leiner, and E.G. Pamer. 2015. "Innate Immune Defenses Mediated by Two Ilc Subsets Are Critical for Protection against Acute Clostridium Difficile Infection." *Cell Host and Microbe* 18 (1): 27–37. https://doi.org/10.1016/j.chom.2015.06.011.

Abt, M.C., P.T. McKenney, and E.G. Pamer. 2016. "Clostridium Difficile Colitis: Pathogenesis and Host Defence." *Nature Reviews Microbiology* 14 (10): 609–20. https://doi.org/10.1038/nrmicro.2016.108.

Adamu B.O., and T.D. Lawley. 2013. "Bacteriotherapy for the Treatment of Intestinal Dysbiosis Caused by Clostridium Difficile Infection." *Current Opinion in Microbiology* 16 (5): 596–601. http://dx.doi.org/10.1016/j.mib.2013.06.009.

Ahn, J., R. Sinha, Z. Pei, C. Dominianni, J. Wu, J. Shi, J.J. Goedert, R.B. Hayes, and L. Yang. 2013. "Human Gut Microbiome and Risk for Colorectal Cancer." *Journal of the National Cancer Institute* 105 (24): 1907–11. https://doi.org/10.1093/jnci/djt300.

Arthur, J.C., E. Perez-Chanona, M. Muhlbauer, S. Tomkovich, J.M. Uronis, T.-J. Fan, B.J. Campbell, *et al*. 2012. "Intestinal Inflammation Targets Cancer-Inducing Activity of the Microbiota." *Science* 338 (6103): 120–23. https://doi.org/10.1126/science.1224820.

Awad, M.M., P.A. Johanesen, G.P. Carter, E. Rose, and D. Lyras. 2015. "Clostridium Difficile Virulence Factors: Insights into an Anaerobic Spore-Forming Pathogen." *Gut Microbes* 5 (5): 579–93. https://doi.org/10.4161/19490976.2014.969632.

Baban, S.T., S.A. Kuehne, A. Barketi-Klai, S.T. Cartman, M.L. Kelly, K.R. Hardie, I. Kansau, A. Collignon, and N.P. Minton. 2013. "The Role of Flagella in Clostridium Difficile Pathogenesis: Comparison between a Non-Epidemic and an Epidemic Strain." *PLoS ONE* 8 (9). https://doi.org/10.1371/journal.pone.0073026.

Bacci, S., K. Mølbak, M.K. Kjeldsen, and K.E.P. Olsen. 2011. "Binary Toxin and Death after Clostridium Difficile Infection." *Emerging Infectious Diseases* 17 (6): 976–82. https://doi.org/10.3201/eid/1706.101483.

Baker, A.M., W. Cross, K. Curtius, I. Al Bakir, C.H.R. Choi, H.L. Davis, D. Temko, *et al*. 2019. "Evolutionary History of Human Colitis-Associated Colorectal Cancer." *Gut* 68 (6): 985–95. https://doi.org/10.1136/gutjnl-2018-316191.

Bartlett, G., A.B. Onderdonk, R.L. Cisneros, and D.L. Kasper. 1977. "Clindamycin-Associated Colitis Due to a Toxin-Producing Species of Clostridium in Hamsters." *Journal of Infectious Diseases* 136 (5): 701–5. https://doi.org/10.1093/infdis/136.5.701.

Bartlett, J.G., N. Moon, T.W. Chang, N. Taylor, and A.B. Onderdonk. 1978. "Role of Clostridium Difficile in Antibiotic-Associated Pseudomembranous Colitis." *Gastroenterology* 75 (5): 778–82. https://doi.org/10.1016/0016-5085(78)90457-2.

Baxter, N.T., J.P. Zackular, G.Y. Chen, and P.D. Schloss. 2014. "Structure of the Gut Microbiome

Following Colonization with Human Feces Determines Colonic Tumor Burden." *Microbiome* 2 (1): 1–11. https://doi.org/10.1186/2049-2618-2-20.

Behnsen, J., S. Jellbauer, C.P. Wong, R.A. Edwards, M.D. George, W. Ouyang, and M. Raffatellu. 2014. "The Cytokine IL-22 Promotes Pathogen Colonization by Suppressing Related Commensal Bacteria." *Immunity* 40 (2): 262–73. https://doi.org/10.1016/j.immuni.2014.01.003.

Benatar, T., M.Y. Cao, Y. Lee, J. Lightfoot, N. Feng, X. Gu, V. Lee, *et al*. 2010. "IL-17E, a Proinflammatory Cytokine, Has Antitumor Efficacy against Several Tumor Types in Vivo." *Cancer Immunology, Immunotherapy* 59 (6): 805–17. https://doi.org/10.1007/s00262-009-0802-8.

Bergonzelli, G.E., D. Granato, R.D. Pridmore, L.F. Marvin-Guy, D. Donnicola, and I.E. Corthésy-Theulaz. 2006. "GroEL of Lactobacillus Johnsonii La1 (NCC 533) Is Cell Surface Associated: Potential Role in Interactions with the Host and the Gastric Pathogen Helicobacter Pylori." *Infection and Immunity* 74 (1): 425–34. https://doi.org/10.1128/IAI.74.1.425-434.2006.

Berry, C.E., K.A. Davies, D.W. Owens, and M.H. Wilcox. 2017. "Is There a Relationship between the Presence of the Binary Toxin Genes in Clostridium Difficile Strains and the Severity of C. Difficile Infection (CDI)?" *European Journal of Clinical Microbiology and Infectious Diseases* 36 (12): 2405–15. https://doi.org/10.1007/s10096-017-3075-8.

Best, E.L., J. Freeman, and M.H. Wilcox. 2012. "Models for the Study of Clostridium Difficile Infection." *Gut Microbes* 3 (2): 145–67. https://doi.org/10.4161/gmic.19526.

Bielanski, A., and J. Haber. 2020. "Structure, Function and Diversity of the Healthy Human Microbiome." *Oxygen in Catalysis* 486 (7402): 330–35. https://doi.org/10.1201/9781482293289-58.

Bray, F., J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, and A. Jemal. 2018. "Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries." *CA: A Cancer Journal for Clinicians* 68 (6): 394–424. https://doi.org/10.3322/caac.21492.

Brenner, H., M. Kloor, and C.P. Pox. 2014. "Colorectal Cancer." In *The Lancet*. https://doi.org/10.1016/S0140-6736(13)61649-9.

Britton, R.A., and V.B. Young. 2014. "Role of the Intestinal Microbiota in Resistance to Colonization by Clostridium Difficile." *Gastroenterology* 146 (6): 1547–53. https://doi.org/10.1053/j.gastro.2014.01.059.

BT, M., K. Tamai, and X. He. 2009. "Wnt/Beta-Catenin Signaling: Components, Mechanisms, and Diseases." *Dev Cell* 17: 9–26. http://dx.doi.org/10.1016/j.devcel.2009.06.016.

Buffie, C.G., I. Jarchum, M. Equinda, L. Lipuma, A. Gobourne, A. Viale, C. Ubeda, J. Xavier, and E.G. Pamer. 2012. "Profound Alterations of Intestinal Microbiota Following a Single Dose of Clindamycin Results in Sustained Susceptibility to Clostridium Difficile-Induced Colitis." *Infection and Immunity* 80 (1): 62–73. https://doi.org/10.1128/IAI.05496-11.

Bullman, S., C.S. Pedamallu, E. Sicinska, T.E. Clancy, X. Zhang, D. Cai, D. Neuberg, *et al*. 2017.

“Analysis of Fusobacterium Persistence and Antibiotic Response in Colorectal Cancer.” *Science*. https://doi.org/10.1126/science.aal5240.

Buonomo, E.L., C.A. Cowardin, M.G. Wilson, M.M. Saleh, P. Pramoonjago, and W.A. Petri. 2016. “Microbiota-Regulated IL-25 Increases Eosinophil Number to Provide Protection during Clostridium Difficile Infection.” *Cell Reports* 16 (2): 432–43. https://doi.org/10.1016/j.celrep.2016.06.007.

Buonomo, E.L., and W.A. Petri. 2015. “The Bug Stops Here: Innate Lymphoid Cells in Clostridium Difficile Infection.” *Cell Host and Microbe* 18 (1): 5–6. https://doi.org/10.1016/j.chom.2015.06.015.

Buonomo, E.L., and W.A. Petri. 2016. “The Microbiota and Immune Response during Clostridium Difficile Infection.” *Anaerobe* 41: 79–84. https://doi.org/10.1016/j.anaerobe.2016.05.009.

Burgner, D., S. Siarakas, G. Eagles, A. Mccarthy, R. Bradbury, and M. Stevens. 1997. “A Prospective Study of Clostridium Difficile Infection Colonization in Pediatric Oncology Patients.” *Pediatric Infectious Disease Journal*. https://doi.org/10.1097/00006454-199712000-00006.

Carter, G.P., J.I. Rood, and D. Lyras. 2010. “The Role of Toxin A and Toxin B in Clostridium Difficile-Associated Disease: Past and Present Perspectives.” *Gut Microbes* 1 (1): 58–64. https://doi.org/10.4161/gmic.1.1.10768.

Castellarin, M., R.L. Warren, J.D. Freeman, L. Dreolini, M. Krzywinski, J. Strauss, R. Barnes, *et al*. 2012. “Fusobacterium Nucleatum Infection Is Prevalent in Human Colorectal Carcinoma.” *Genome Research* 22 (2): 299–306. https://doi.org/10.1101/gr.126516.111.

Chandrasekaran, R., and D.B. Lacy. 2017. “The Role of Toxins in Clostridium Difficile Infection.” *FEMS Microbiology Reviews* 41 (6): 723–50. https://doi.org/10.1093/femsre/fux048.

Chen, J., J.C. Domingue, and C.L. Sears. 2017. “Microbiota Dysbiosis in Select Human Cancers: Evidence of Association and Causality.” *Seminars in Immunology* 32 (June): 25–34. https://doi.org/10.1016/j.smim.2017.08.001.

Chen, P., L. Tao, T. Wang, J. Zhang, A. He, K. ho Lam, Z. Liu, *et al*. 2018. “Structural Basis for Recognition of Frizzled Proteins by Clostridium Difficile Toxin B.” *Science*. https://doi.org/10.1126/science.aar1999.

Chen, X., K. Katchar, J.D. Goldsmith, N. Nanthakumar, A. Cheknis, D.N. Gerding, and C.P. Kelly. 2008. “A Mouse Model of Clostridium Difficile-Associated Disease.” *Gastroenterology* 135 (6): 1984–92. https://doi.org/10.1053/j.gastro.2008.09.002.

Chiang, J.Y.L. 2004. “Regulation of Bile Acid Synthesis: Pathways, Nuclear Receptors, and Mechanisms.” *Journal of Hepatology* 40 (3): 539–51. https://doi.org/10.1016/j.jhep.2003.11.006.

Chopra, T., G.J. Alangaden, and P. Chandrasekar. 2010. “Clostridium Difficile Infection in Cancer Patients and Hematopoietic Stem Cell Transplant Recipients.” *Expert Review of Anti-Infective Therapy*. https://doi.org/10.1586/eri.10.95.

Chumbler, N.M., M.A. Farrow, L.A. Lapierre, J.L. Franklin, and D. Borden. 2016. "Clostridium Difficile Toxins TcdA and TcdB Cause Colonic Tissue." *Infection and Immunity* 84 (10): 2871–77. https://doi.org/10.1128/IAI.00583-16.

Chung, L., E. Thiele Orberg, A.L. Geis, J.L. Chan, K. Fu, C.E. DeStefano Shields, C.M. Dejea, *et al*. 2018. "Bacteroides Fragilis Toxin Coordinates a Pro-Carcinogenic Inflammatory Cascade via Targeting of Colonic Epithelial Cells." *Cell Host and Microbe* 23 (2): 203-214.e5. https://doi.org/10.1016/j.chom.2018.01.007.

Colnot, S., M. Niwa-Kawakita, G. Hamard, C. Godard, S. Le Plenier, C. Houbron, B. Romagnolo, *et al*. 2004. "Colorectal Cancers in a New Mouse Model of Familial Adenomatous Polyposis: Influence of Genetic and Environmental Modifiers." *Laboratory Investigation* 84 (12): 1619–30. https://doi.org/10.1038/labinvest.3700180.

Costerton, W., R. Veeh, M. Shirtliff, M. Pasmore, C. Post, and G. Ehrlich. 2003. "The Application of Biofilm Science to the Study and Control of Chronic Bacterial Infections." *Journal of Clinical Investigation* 112 (10): 1466–77. https://doi.org/10.1172/JCI200320365.

Coutzac, C., J.M. Jouniaux, A. Paci, J. Schmidt, D. Mallardo, A. Seck, V. Asvatourian, *et al*. 2020. "Systemic Short Chain Fatty Acids Limit Antitumor Effect of CTLA-4 Blockade in Hosts with Cancer." *Nature Communications* 11 (1): 1–13. https://doi.org/10.1038/s41467-020-16079-x.

Cowardin, C.A., E.L. Buonomo, M.M. Saleh, M.G. Wilson, S.L. Burgess, S.A. Kuehne, C. Schwan, *et al*. 2016. "The Binary Toxin CDT Enhances Clostridium Difficile Virulence by Suppressing Protective Colonic Eosinophilia." *Nature Microbiology* 1 (8): 1–10. https://doi.org/10.1038/nmicrobiol.2016.108.

D'Aoust, J., R. Battat, and T. Bessissow. 2017. "Management of Inflammatory Bowel Disease with Clostridium Difficile Infection." *World Journal of Gastroenterology* 23 (27): 4986–5003. https://doi.org/10.3748/wjg.v23.i27.4986.

Daiber, A. 2010. "Redox Signaling (Cross-Talk) from and to Mitochondria Involves Mitochondrial Pores and Reactive Oxygen Species." *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1797: 55–56. https://doi.org/10.1016/j.bbabio.2010.04.181.

Dang, T.H.T., L. De La Riva, R.P. Fagan, E.M. Storck, W.P. Heal, C. Janoir, N.F. Fairweather, and E.W. Tate. 2010. "Chemical Probes of Surface Layer Biogenesis in Clostridium Difficile." *ACS Chemical Biology* 5 (3): 279–85. https://doi.org/10.1021/cb9002859.

Dapa, T., R. Leuzzi, Y.K. Ng, S.T. Baban, R. Adamo, S.A. Kuehne, M. Scarselli, *et al*. 2013. "Multiple Factors Modulate Biofilm Formation by the Anaerobic Pathogen Clostridium Difficile." *Journal of Bacteriology* 195 (3): 545–55. https://doi.org/10.1128/JB.01980-12.

Dapa, T., and M. Unnikrishnan. 2013. "Biofilm Formation by Clostridium Difficile." *Gut Microbes* 4 (5). https://doi.org/10.4161/gutm.25862.

Darkoh, C., H.L. Dupont, S.J. Norris, and H.B. Kaplan. 2015. "Toxin Synthesis by Clostridium Difficile Is Regulated through Quorum Signaling." *MBio* 6 (2): 1–10. https://doi.org/10.1128/mBio.02569-14.

Deitrick, J., and W.M. Pruitt. 2016. “Wnt/β Catenin-Mediated Signaling Commonly Altered in Colorectal Cancer.” *Progress in Molecular Biology and Translational Science* 144: 49–68. https://doi.org/10.1016/bs.pmbts.2016.09.010.

Dejea, C.M., E.C. Wick, E.M. Hechenbleikner, J.R. White, J.L. Mark Welch, B.J. Rossetti, S.N. Peterson, *et al*. 2014. “Microbiota Organization Is a Distinct Feature of Proximal Colorectal Cancers.” *Proceedings of the National Academy of Sciences* 111 (51): 18321–26. https://doi.org/10.1073/pnas.1406199111.

Dominguez-Bello, M.G., F. Godoy-Vitorino, R. Knight, and M.J. Blaser. 2019. “Role of the Microbiome in Human Development.” *Gut* 68 (6): 1108–14. https://doi.org/10.1136/gutjnl-2018-317503.

Drewes, J.L., F. Housseau, and C.L. Sears. 2016. “Sporadic Colorectal Cancer: Microbial Contributors to Disease Prevention, Development and Therapy.” *British Journal of Cancer* 115 (3): 273–80. https://doi.org/10.1038/bjc.2016.189.

Drewes, J.L., J.R. White, C.M. Dejea, P. Fathi, T. Iyadorai, J. Vadivelu, A.C. Roslani, *et al*. 2017. “High-Resolution Bacterial 16S RRNA Gene Profile Meta-Analysis and Biofilm Status Reveal Common Colorectal Cancer Consortia.” *Npj Biofilms and Microbiomes*. https://doi.org/10.1038/s41522-017-0040-3.

Dunne, C., B. Dolan, and M. Clyne. 2014. “Factors That Mediate Colonization of the Human Stomach by Helicobacter Pylori.” *World Journal of Gastroenterology* 20 (19): 5610–24. https://doi.org/10.3748/wjg.v20.i19.5610.

Elinav, E., R. Nowarski, C.A. Thaiss, B. Hu, C. Jin, and R.A. Flavell. 2013. “Inflammation-Induced Cancer: Crosstalk between Tumours, Immune Cells and Microorganisms.” *Nature Reviews Cancer* 13 (11): 759–71. https://doi.org/10.1038/nrc3611.

Engevik, M.A., M.B. Yacyshyn, K.A. Engevik, J. Wang, B. Darien, D.J. Hassett, B.R. Yacyshyn, and R.T. Worrell. 2014. “Human Clostridium Difficile Infection: Altered Mucus Production and Composition.” *American Journal of Physiology - Gastrointestinal and Liver Physiology*. https://doi.org/10.1152/ajpgi.00091.2014.

Erben, U., C. Loddenkemper, K. Doerfel, S. Spieckermann, D. Haller, M.M. Heimesaat, M. Zeitz, B. Siegmund, and A.A. Ku¨hl. 2014. “A Guide to Histomorphological Evaluation of Intestinal Inflammation in Mouse Models.” *International Journal of Clinical and Experimental Pathology* 7 (8): 4557–76.

Farrow, M.A., N.M. Chumbler, L.A. Lapierre, J.L. Franklin, S.A. Rutherford, J.R. Goldenring, and D.B. Lacy. 2013. “Clostridium Difficile Toxin B-Induced Necrosis Is Mediated by the Host Epithelial Cell NADPH Oxidase Complex.” *Proceedings of the National Academy of Sciences of the United States of America* 110 (46): 18674–79. https://doi.org/10.1073/pnas.1313658110.

Feng, Q., S. Liang, H. Jia, A. Stadlmayr, L. Tang, Z. Lan, D. Zhang, *et al*. 2015. “Gut Microbiome Development along the Colorectal Adenoma-Carcinoma Sequence.” *Nature Communications* 6. https://doi.org/10.1038/ncomms7528.

Fettucciari, K., L. Macchioni, M. Davidescu, P. Scarpelli, C. Palumbo, L. Corazzi, A. Marchegiani,

et al. 2018. "Clostridium Difficile Toxin B Induces Senescence in Enteric Glial Cells: A Potential New Mechanism of Clostridium Difficile Pathogenesis." *Biochimica et Biophysica Acta - Molecular Cell Research* 1865 (12): 1945–58. https://doi.org/10.1016/j.bbamcr.2018.10.007.

Flynn, K.J., N.T. Baxter, and P.D. Schloss. 2016. "Metabolic and Community Synergy of Oral Bacteria in Colorectal Cancer." *MSphere* 1 (3): 1–6. https://doi.org/10.1128/msphere.00102-16.

Fodde, R., R. Smits, and H. Clevers. 2001. "APC, Signal Transduction and Genetic Instability in Colorectal Cancer." *Nature Reviews Cancer* 1 (1): 55–67. https://doi.org/10.1038/35094067.

Frisbee, A.L., M.M. Saleh, M.K. Young, J.L. Leslie, M.E. Simpson, M.M. Abhyankar, C.A. Cowardin, *et al*. 2019. "IL-33 Drives Group 2 Innate Lymphoid Cell-Mediated Protection during Clostridium Difficile Infection." *Nature Communications* 10 (1): 1–13. https://doi.org/10.1038/s41467-019-10733-9.

Fukugaiti, M.H., A. Ignacio, M.R. Fernandes, U. Ribeiro, V. Nakano, and M.J. Avila-Campos. 2015. "High Occurrence of Fusobacterium Nucleatum and Clostridium Difficile in the Intestinal Microbiota of Colorectal Carcinoma Patients." *Brazilian Journal of Microbiology*. https://doi.org/10.1590/S1517-838246420140665.

Gagliani, N., B. Hu, S. Huber, E. Elinav, and R.A. Flavell. 2014. "The Fire within: Microbes Inflame Tumors." *Cell*. https://doi.org/10.1016/j.cell.2014.03.006.

Galdys, A.L., J.S. Nelson, K.A. Shutt, J.L. Schlackman, D.L. Pakstis, A.W. Pasculle, J.W. Marsh, L.H. Harrison, and S.R. Curry. 2014. "Prevalence and Duration of Asymptomatic Clostridium Difficile Carriage among Healthy Subjects in Pittsburgh, Pennsylvania." *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.00222-14.

Galperin, M.Y., V. Brover, I. Tolstoy, and N. Yutin. 2016. "Phylogenomic Analysis of the Family Peptostreptococcaceae (Clostridium Cluster Xi) and Proposal for Reclassification of Clostridium Litorale (Fendrich *et al*. 1991) and Eubacterium Acidaminophilum (Zindel *et al*. 1989) as Peptoclostridium Litorale Gen. Nov." *International Journal of Systematic and Evolutionary Microbiology* 66 (12): 5506–13. https://doi.org/10.1099/ijsem.0.001548.

Gao, Z., B. Guo, R. Gao, Q. Zhu, and H. Qin. 2015. "Microbiota Disbiosis Is Associated with Colorectal Cancer." *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2015.00020.

Garrett, W.S. 2019. "The Gut Microbiota and Colon Cancer." *Science* 364 (6446): 1133–35. https://doi.org/10.1126/science.aaw2367.

Geiger, T.L., M.C. Abt, G. Gasteiger, M.A. Firth, M.H. O'Connor, C.D. Geary, T.E. O'Sullivan, *et al*. 2014. "Nfil3 Is Crucial for Development of Innate Lymphoid Cells and Host Protection against Intestinal Pathogens." *Journal of Experimental Medicine* 211 (9): 1723–31. https://doi.org/10.1084/jem.20140212.

Gerding, D.N., S. Johnson, M. Rupnik, and K. Aktories. 2014. " Clostridium Difficile Binary Toxin CDT ." *Gut Microbes* 5 (1): 15–27. https://doi.org/10.4161/gmic.26854.

Goodman, B., and H. Gardner. 2018. "The Microbiome and Cancer." *Journal of Pathology*.

https://doi.org/10.1002/path.5047.

Goodwin, A.C., C.E.D. Shields, S. Wu, D.L. Huso, X. Wu, T.R. Murray-Stewart, A. Hacker-Prietz, *et al*. 2011. “Polyamine Catabolism Contributes to Enterotoxigenic Bacteroides Fragilis-Induced Colon Tumorigenesis.” *Proceedings of the National Academy of Sciences* 108 (37): 15354–59. https://doi.org/10.1073/pnas.1010203108.

Grivennikov, S.I., K. Wang, D. Mucida, C.A. Stewart, B. Schnabl, D. Jauch, K. Taniguchi, *et al*. 2012. “Adenoma-Linked Barrier Defects and Microbial Products Drive IL-23/IL-17-Mediated Tumour Growth.” *Nature* 491 (7423): 254–58. https://doi.org/10.1038/nature11465.

Guery, B., F. Menichetti, V.J. Anttila, N. Adomakoh, J.M. Aguado, K. Bisnauthsing, A. Georgopali, *et al*. 2018. “Extended-Pulsed Fidaxomicin versus Vancomycin for Clostridium Difficile Infection in Patients 60 Years and Older (EXTEND): A Randomised, Controlled, Open-Label, Phase 3b/4 Trial.” *The Lancet Infectious Diseases* 18 (3): 296–307. https://doi.org/10.1016/S1473-3099(17)30751-X.

Guh, A.Y., Y. Mu, L.G. Winston, H. Johnston, D. Olson, M.M. Farley, L.E. Wilson, *et al*. 2020. “ Trends in U.S. Burden of Clostridioides Difficile Infection and Outcomes .” *New England Journal of Medicine* 382 (14): 1320–30. https://doi.org/10.1056/nejmoa1910215.

Hall-Stoodley, L., J. Costerton, and P. Stoodley. 2004. “Bacterial Biofilms: From the Natural Environment to Infectious Diseases.” *Nature Reviews. Microbiology*. https://doi.org/10.1038/nrmicro821.

HALL, I., and E. O’Toole. 1935. “Intestinal Flora in New-Born Infants: With a Description of a New Pathogenic Anaerobe, Bacillus Difficilis.” *Archives of Pediatrics and Adolescent Medicine* 49 (2): 390–402. https://doi.org/10.1001/archpedi.1935.01970020105010.

Hasegawa, M., N. Kamada, Y. Jiao, M.Z. Liu, G. Núñez, and N. Inohara. 2012. “ Protective Role of Commensals against Clostridium Difficile Infection via an IL-1β–Mediated Positive-Feedback Loop .” *The Journal of Immunology* 189 (6): 3085–91. https://doi.org/10.4049/jimmunol.1200821.

Hasegawa, M., S. Yada, M.Z. Liu, N. Kamada, R. Muñoz-Planillo, N. Do, G. Núñez, and N. Inohara. 2014. “Interleukin-22 Regulates the Complement System to Promote Resistance against Pathobionts after Pathogen-Induced Intestinal Damage.” *Immunity* 41 (4): 620–32. https://doi.org/10.1016/j.immuni.2014.09.010.

Hinoi, T., A. Akyol, B.K. Theisen, D.O. Ferguson, J.K. Greenson, B.O. Williams, K.R. Cho, and E.R. Fearon. 2007. “Mouse Model of Colonic Adenoma-Carcinoma Progression Based on Somatic Apc Inactivation.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-07-2735.

Howerton, A., N. Ramirez, and E. Abel-Santos. 2011. “Mapping Interactions between Germinants and Clostridium Difficile Spores.” *Journal of Bacteriology* 193 (1): 274–82. https://doi.org/10.1128/JB.00980-10.

Hurtado, C.G., F. Wan, F. Housseau, and C.L. Sears. 2018. “Roles for Interleukin 17 and Adaptive Immunity in Pathogenesis of Colorectal Cancer.” *Gastroenterology* 155 (6): 1706–15. https://doi.org/10.1053/j.gastro.2018.08.056.

Hutton, M.L., K.E. Mackin, A. Chakravorty, and D. Lyras. 2014. "Small Animal Models for the Study of Clostridium Difficile Disease Pathogenesis." *FEMS Microbiology Letters* 352 (2): 140–49. https://doi.org/10.1111/1574-6968.12367.

Huycke, M.M., V. Abrams, and D.R. Moore. 2002. "Enterococcus Faecalis Produces Extracellular Superoxide and Hydrogen Peroxide That Damages Colonic Epithelial Cell DNA." *Carcinogenesis* 23 (3): 529–36. https://doi.org/10.1093/carcin/23.3.529.

Janoir, C., S. Péchiné, C. Grosdidier, and A. Collignon. 2007. "Cwp84, a Surface-Associated Protein of Clostridium Difficile, Is a Cysteine Protease with Degrading Activity on Extracellular Matrix Proteins." *Journal of Bacteriology* 189 (20): 7174–80. https://doi.org/10.1128/JB.00578-07.

Jasperson, K.W., T.M. Tuohy, D.W. Neklason, and R.W. Burt. 2010. "Hereditary and Familial Colon Cancer." *Gastroenterology*. https://doi.org/10.1053/j.gastro.2010.01.054.

Johnson, C.H., C.M. Dejea, D. Edler, L.T. Hoang, A.F. Santidrian, B.H. Felding, J. Ivanisevic, *et a*l. 2015. "Metabolism Links Bacterial Biofilms and Colon Carcinogenesis." *Cell Metabolism* 21 (6): 891–97. https://doi.org/10.1016/j.cmet.2015.04.011.

Johnston, P.F., D.N. Gerding, and K.L. Knight. 2014. "Protection from Clostridium Difficile Infection in CD4 T Cell- and Polymeric Immunoglobulin Receptor-Deficient Mice." *Infection and Immunity* 82 (2): 522–31. https://doi.org/10.1128/IAI.01273-13.

Jones, B. V., M. Begley, C. Hill, C.G.M. Gahan, and J.R. Marchesi. 2008. "Functional and Comparative Metagenomic Analysis of Bile Salt Hydrolase Activity in the Human Gut Microbiome." *Proceedings of the National Academy of Sciences of the United States of America* 105 (36): 13580–85. https://doi.org/10.1073/pnas.0804437105.

Just, I., J. Selzer, M. Wilm, C. Von Eichel-Streiber, M. Mann, and K. Aktories. 1995. "Glucosylation of Rho Proteins by Clostridium Difficile Toxin B." *Nature*. https://doi.org/10.1038/375500a0.

Kachrimanidou, M., and E. Tsintarakis. 2020. "Insights into the Role of Human Gut Microbiota in Clostridioides Difficile Infection." *Microorganisms* 8 (2): 8–10. https://doi.org/10.3390/microorganisms8020200.

Karen, C.C., and B.G. John. 2011. "Biology of Clostridium Difficile: Implications for Epidemiology and Diagnosis." *Annual Review of Microbiology* 65: 501–21. https://doi.org/10.1146/annurev-micro-090110-102824.

Kelly, C.P., S. Becker, J.K. Linevsky, M.A. Joshi, J.C. O'Keane, B.F. Dickey, J.T. LaMont, and C. Pothoulakis. 1994. "Neutrophil Recruitment in Clostridium Difficile Toxin A Enteritis in the Rabbit." *Journal of Clinical Investigation* 93 (3): 1257–65. https://doi.org/10.1172/JCI117080.

Kelly, C.P., S. Keates, D. Siegenberg, J.K. Linevsky, C. Pothoulakis, and H.R. Brady. 1994. "IL-8 Secretion and Neutrophil Activation by HT-29 Colonic Epithelial Cells." *American Journal of Physiology - Gastrointestinal and Liver Physiology* 267 (6 30-6): 991–97. https://doi.org/10.1152/ajpgi.1994.267.6.g991.

Kelly, C.P., and L. Kyne. 2011. "The Host Immune Response to Clostridium Difficile." *Journal of Medical Microbiology* 60 (8): 1070–79. https://doi.org/10.1099/jmm.0.030015-0.

Kelly, C.P., and J.T. LaMont. 2008. "Clostridium Difficile — More Difficult Than Ever ." *New*

England Journal of Medicine. https://doi.org/10.1056/nejmra0707500.

Killgore, G., A. Thompson, S. Johnson, J. Brazier, E. Kuijper, J. Pepin, E.H. Frost, *et al*. 2008. "Comparison of Seven Techniques for Typing International Epidemic Strains of Clostridium Difficile: Restriction Endonuclease Analysis, Pulsed-Field Gel Electrophoresis, PCR-Ribotyping, Multilocus Sequence Typing, Multilocus Variable-Number Tandem-Repeat An." *Journal of Clinical Microbiology* 46 (2): 431–37. https://doi.org/10.1128/JCM.01484-07.

Kirby, J. 2011. "The Pathogenesis of Clostridium Difficile Infection." *The Journal of Antimicrobial Chemotherapy* 41 Suppl C (2): 13–19. https://doi.org/10.1016/j.molimm.2014.09.005.The.

Kostic, A.D., E. Chun, L. Robertson, J.N. Glickman, C.A. Gallini, M. Michaud, T.E. Clancy, *et al*. 2013. "Fusobacterium Nucleatum Potentiates Intestinal Tumorigenesis and Modulates the Tumor-Immune Microenvironment." *Cell Host and Microbe*. https://doi.org/10.1016/j.chom.2013.07.007.

Kostic, A.D., D. Gevers, C.S. Pedamallu, M. Michaud, F. Duke, A.M. Earl, A.I. Ojesina, *et al*. 2012. "Genomic Analysis Identifies Association of Fusobacterium with Colorectal Carcinoma." *Genome Research* 22 (2): 292–98. https://doi.org/10.1101/gr.126573.111.

Kuehne, S.A., S.T. Cartman, J.T. Heap, M.L. Kelly, A. Cockayne, and N.P. Minton. 2010. "The Role of Toxin A and Toxin B in Clostridium Difficile Infection." *Nature* 467 (7316): 711–13. https://doi.org/10.1038/nature09397.

Kuehne, S.A., M.M. Collery, M.L. Kelly, S.T. Cartman, A. Cockayne, and N.P. Minton. 2014. "Importance of Toxin a, Toxin b, and Cdt in Virulence of an Epidemic Clostridium Difficile Strain." *Journal of Infectious Diseases* 209 (1): 83–86. https://doi.org/10.1093/infdis/jit426.

Kyne, L., M. Warny, A. Qamar, and C.P. Kelly. 2002. " Asymptomatic Carriage of Clostridium Difficile and Serum Levels of IgG Antibody against Toxin A ." *New England Journal of Medicine*. https://doi.org/10.1056/nejm200002103420604.

la Riva, L. de, S.E. Willing, E.W. Tate, and N.F. Fairweather. 2011. "Roles of Cysteine Proteases Cwp84 and Cwp13 in Biogenesis of the Cell Wall of Clostridium Difficile." *Journal of Bacteriology* 193 (13): 3276–85. https://doi.org/10.1128/JB.00248-11.

Lawley, T.D., N.J. Croucher, L. Yu, S. Clare, M. Sebaihia, D. Goulding, D.J. Pickard, *et al*. 2009. "Proteomic and Genomic Characterization of Highly Infectious Clostridium Difficile 630 Spores." *Journal of Bacteriology* 191 (17): 5377–86. https://doi.org/10.1128/JB.00597-09.

Lawley, T.D., and V.B. Young. 2013. "Murine Models to Study Clostridium Difficile Infection and Transmission." *Anaerobe* 24: 94–97. https://doi.org/10.1016/j.anaerobe.2013.09.008.

Lawson, P.A., D.M. Citron, K.L. Tyrrell, and S.M. Finegold. 2016. "Reclassification of Clostridium Difficile as Clostridioides Difficile (Hall and O'Toole 1935) Prévot 1938." *Anaerobe* 40: 95–99. https://doi.org/10.1016/j.anaerobe.2016.06.008.

Lessa, F.C., Y. Mu, W.M. Bamberg, Z.G. Beldavs, G.K. Dumyati, J.R. Dunn, M.M. Farley, *et al*. 2015. " Burden of Clostridium Difficile Infection in the United States ." *New England Journal of Medicine* 372 (9): 825–34. https://doi.org/10.1056/nejmoa1408913.

Li, L., X. Li, W. Zhong, M. Yang, M. Xu, Y. Sun, J. Ma, *et al*. 2019. "Gut Microbiota from Colorectal Cancer Patients Enhances the Progression of Intestinal Adenoma in Apcmin/+ Mice." *EBioMedicine* 48: 301–15. https://doi.org/10.1016/j.ebiom.2019.09.021.

Linevsky, J.K., C. Pothoulakis, S. Keates, M. Warny, A.C. Keates, J.T. Lamont, and C.P. Kelly. 1997. "IL-8 Release and Neutrophil Activation by Clostridium Difficile Toxin- Exposed Human Monocytes." *American Journal of Physiology - Gastrointestinal and Liver Physiology* 273 (6 36-6): 1333–40. https://doi.org/10.1152/ajpgi.1997.273.6.g1333.

Lloyd-Price, J., G. Abu-Ali, and C. Huttenhower. 2016. "The Healthy Human Microbiome." *Genome Medicine* 8 (1): 1–11. https://doi.org/10.1186/s13073-016-0307-y.

Long, X., C.C. Wong, L. Tong, E.S.H. Chu, C. Ho Szeto, M.Y.Y. Go, O.O. Coker, *et al*. 2019. "Peptostreptococcus Anaerobius Promotes Colorectal Carcinogenesis and Modulates Tumour Immunity." *Nature Microbiology* 4 (12): 2319–30. https://doi.org/10.1038/s41564-019-0541-3.

Loo, V.G., L. Poirier, M.A. Miller, M. Oughton, M.D. Libman, S. Michaud, A.-M. Bourgault, *et al*. 2005. " A Predominantly Clonal Multi-Institutional Outbreak of Clostridium Difficile – Associated Diarrhea with High Morbidity and Mortality ." *New England Journal of Medicine* 353 (23): 2442–49. https://doi.org/10.1056/nejmoa051639.

Louie, T.J., M.A. Miller, K.M. Mullane, K. Weiss, A. Lentnek, Y. Golan, S. Gorbach, P. Sears, and Y.-K. Shue. 2011. " Fidaxomicin versus Vancomycin for Clostridium Difficile Infection ." *New England Journal of Medicine* 364 (5): 422–31. https://doi.org/10.1056/nejmoa0910812.

Louis, P., G.L. Hold, and H.J. Flint. 2014. "The Gut Microbiota, Bacterial Metabolites and Colorectal Cancer." *Nature Reviews Microbiology* 12 (10): 661–72. https://doi.org/10.1038/nrmicro3344.

Lyras, D., J.R. O'Connor, P.M. Howarth, S.P. Sambol, G.P. Carter, T. Phumoonna, R. Poon, *et al*. 2009. "Toxin B Is Essential for Virulence of Clostridium Difficile." *Nature*. https://doi.org/10.1038/nature07822.

Marsh, J.W., R. Arora, J.L. Schlackman, K.A. Shutt, S.R. Curry, and L.H. Harrison. 2012. "Association of Relapse of Clostridium Difficile Disease with BI/NAP1/027." *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.02291-12.

Martin-Verstraete, I., J. Peltier, and B. Dupuy. 2016. "The Regulatory Networks That Control Clostridium Difficile Toxin Synthesis." *Toxins* 8 (5): 1–24. https://doi.org/10.3390/toxins8050153.

Maseda, D., J.P. Zackular, B. Trindade, L. Kirk, J.L. Roxas, L.M. Rogers, M.K. Washington, *et al*. 2019. "Nonsteroidal Anti-Inflammatory Drugs Alter the Microbiota and Exacerbate Clostridium Difficile Colitis While Dysregulating the Inflammatory Response." *MBio* 10 (1): 1–18. https://doi.org/10.1128/mBio.02282-18.

Matson, V., J. Fessler, R. Bao, T. Chongsuwat, Y. Zha, M.L. Alegre, J.J. Luke, and T.F. Gajewski. 2018. "The Commensal Microbiome Is Associated with Anti-PD-1 Efficacy in Metastatic Melanoma Patients." *Science* 359 (6371): 104–8. https://doi.org/10.1126/science.aao3290.

McDonald, L.C., D.N. Gerding, S. Johnson, J.S. Bakken, K.C. Carroll, S.E. Coffin, E.R. Dubberke, *et al*. 2018. "Clinical Practice Guidelines for Clostridium Difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA)." *Clinical Infectious Diseases*. https://doi.org/10.1093/cid/cix1085.

McDonald, L.C., G.E. Killgore, A. Thompson, R.C. Owens, S. V. Kazakova, S.P. Sambol, S. Johnson, and D.N. Gerding. 2005. " An Epidemic, Toxin Gene–Variant Strain of Clostridium Difficile ." *New England Journal of Medicine*. https://doi.org/10.1056/nejmoa051590.

Metcalf, D.S., and J. Scott Weese. 2011. "Binary Toxin Locus Analysis in Clostridium Difficile." *Journal of Medical Microbiology* 60 (8): 1137–45. https://doi.org/10.1099/jmm.0.028498-0.

Mileto, S.J., T. Jardé, K.O. Childress, J.L. Jensen, A.P. Rogers, G. Kerr, M.L. Hutton, *et al*. 2020. "Clostridioides Difficile Infection Damages Colonic Stem Cells via TcdB, Impairing Epithelial Repair and Recovery from Disease." *Proceedings of the National Academy of Sciences of the United States of America* 117 (14): 8064–73. https://doi.org/10.1073/pnas.1915255117.

Muzny, D.M., M.N. Bainbridge, K. Chang, H.H. Dinh, J.A. Drummond, G. Fowler, C.L. Kovar, *et al*. 2012. "Comprehensive Molecular Characterization of Human Colon and Rectal Cancer." *Nature* 487 (7407): 330–37. https://doi.org/10.1038/nature11252.

Na, X., D. Zhao, H.W. Koon, H. Kim, J. Husmark, M.P. Moyer, C. Pothoulakis, and J.T. Lamont. 2005. "Clostridium Difficile Toxin B Activates the EGF Receptor and the ERK/MAP Kinase Pathway in Human Colonocytes." *Gastroenterology* 128 (4): 1002–11. https://doi.org/10.1053/j.gastro.2005.01.053.

Nougayrède, J.-P., S. Homburg, F. Taieb, M. Boury, E. Brzuszkiewicz, G. Gottschalk, C. Buchrieser, *et al*. 2006. "Escherichia Coli Induces DNA Double-Strand Breaks in Eukaryotic Cells." *Science (New York, N.Y.)* 313 (5788): 848–51. https://doi.org/10.1126/science.1127059.

Ohashi, Y., and T. Fujisawa. 2019. "Analysis of Clostridium Cluster XI Bacteria in Human Feces." *Bioscience of Microbiota, Food and Health* 38 (2): 65–68. https://doi.org/10.12938/bmfh.18-023.

Pawlowski, S.W., G. Calabrese, G.L. Kolling, R. Freire, C. Alcantara Warren, B. Liu, R.B. Sartor, and R.L. Guerrant. 2010. "Murine Model of Clostridium Difficile Infection with Aged Gnotobiotic C57BL/6 Mice and a BI/NAP1 Strain." *Journal of Infectious Diseases* 202 (11): 1708–12. https://doi.org/10.1086/657086.

Powell, S.M., N. Zilz, Y. Beazer-Barclay, T.M. Bryan, S.R. Hamilton, S.N. Thibodeau, B. Vogelstein, and K.W. Kinzler. 1992. "APC Mutations Occur Early during Colorectal Tumorigenesis." *Nature* 359 (6392): 235–37. https://doi.org/10.1038/359235a0.

Price, B., H.E. Larson, and J. Crow. 1979. "Morphology of Experimental Antibiotic-Associated Enterocolitis in the Hamster: A Model for Human Pseudomembranous Colitis and Antibiotic-Associated Diarrhea." *Gut* 20 (6): 467–75. https://doi.org/10.1136/gut.20.6.467.

Prior, K.K., I. Wittig, M.S. Leisegang, J. Groenendyk, N. Weissmann, M. Michalak, P. Jansen-Dürr, A.M. Shah, and R.P. Brandes. 2016. "The Endoplasmic Reticulum Chaperone

Calnexin Is a NADPH Oxidase NOX4 Interacting Protein." *Journal of Biological Chemistry* 291 (13): 7045–59. https://doi.org/10.1074/jbc.M115.710772.

Proctor, L.M., H.H. Creasy, J.M. Fettweis, J. Lloyd-Price, A. Mahurkar, W. Zhou, G.A. Buck, *et al*. 2019. "The Integrative Human Microbiome Project." *Nature* 569 (7758): 641–48. https://doi.org/10.1038/s41586-019-1238-8.

Reeves, A.E., M.J. Koenigsknecht, I.L. Bergin, and V.B. Young. 2012. "Suppression of Clostridium Difficile in the Gastrointestinal Tracts of Germfree Mice Inoculated with a Murine Isolate from the Family Lachnospiraceae." *Infection and Immunity* 80 (11): 3786–94. https://doi.org/10.1128/IAI.00647-12.

Rinttilä, T., A. Kassinen, E. Malinen, L. Krogius, and A. Palva. 2004. "Development of an Extensive Set of 16S RDNA-Targeted Primers for Quantification of Pathogenic and Indigenous Bacteria in Faecal Samples by Real-Time PCR." *Journal of Applied Microbiology* 97 (6): 1166–77. https://doi.org/10.1111/j.1365-2672.2004.02409.x.

Rowan, A.J., H. Lamlum, M. Ilyas, J. Wheeler, J. Straub, A. Papadopoulou, D. Bicknell, W.F. Bodmer, and I.P.M. Tomlinson. 2000. "APC Mutations in Sporadic Colorectal Tumors: A Mutational 'Hotspot' and Interdependence of the 'Two Hits.'" *Proceedings of the National Academy of Sciences of the United States of America* 97 (7): 3352–57. https://doi.org/10.1073/pnas.97.7.3352.

Rubinstein, M.R., X. Wang, W. Liu, Y. Hao, G. Cai, and Y.W. Han. 2013. "Fusobacterium Nucleatum Promotes Colorectal Carcinogenesis by Modulating E-Cadherin/β-Catenin Signaling via Its FadA Adhesin." *Cell Host and Microbe* 14 (2): 195–206. https://doi.org/10.1016/j.chom.2013.07.012.

Rupnik, M., M.H. Wilcox, and D.N. Gerding. 2009. "Clostridium Difficile Infection: New Developments in Epidemiology and Pathogenesis." *Nature Reviews Microbiology*. https://doi.org/10.1038/nrmicro2164.

Ryan, A., M. Lynch, S.M. Smith, S. Amu, H.J. Nel, C.E. McCoy, J.K. Dowling, *et al*. 2011. "A Role for TLR4 in Clostridium Difficile Infection and the Recognition of Surface Layer Proteins." *PLoS Pathogens* 7 (6). https://doi.org/10.1371/journal.ppat.1002076.

Saleh, M.M., and W.A. Petri. 2020. "Type 3 Immunity during Clostridioides Difficile Infection: Too Much of a Good Thing?" *Infection and Immunity* 88 (1): 1–14. https://doi.org/10.1128/IAI.00306-19.

Sandhu, B.K., and S.M. McBride. 2018. "Clostridioides Difficile." *Trends in Microbiology* 26 (12): 1049–50. https://doi.org/10.1016/j.tim.2018.09.004.

Schwabe, R.F., and C. Jobin. 2013. "The Microbiome and Cancer." *Nature Reviews Cancer* 13 (11): 800–812. https://doi.org/10.1038/nrc3610.

Schwan, C., B. Stecher, T. Tzivelekidis, M. Van Ham, M. Rohde, W.D. Hardt, J. Wehland, and K. Aktories. 2009. "Clostridium Difficile Toxin CDT Induces Formation of Microtubule-Based Protrusions and Increases Adherence of Bacteria." *PLoS Pathogens* 5 (10). https://doi.org/10.1371/journal.ppat.1000626.

Sears, C.L., and W.S. Garrett. 2014. "Microbes, Microbiota, and Colon Cancer." *Cell Host and Microbe* 15 (3): 317–28. https://doi.org/10.1016/j.chom.2014.02.007.

Sebaihia, M., B.W. Wren, P. Mullany, N.F. Fairweather, N. Minton, R. Stabler, N.R. Thomson, *et al*. 2006. "The Multidrug-Resistant Human Pathogen Clostridium Difficile Has a Highly Mobile, Mosaic Genome." *Nature Genetics*. https://doi.org/10.1038/ng1830.

Siegel, R.L., S.A. Fedewa, W.F. Anderson, K.D. Miller, J. Ma, P.S. Rosenberg, and A. Jemal. 2017. "Colorectal Cancer Incidence Patterns in the United States, 1974-2013." *Journal of the National Cancer Institute* 109 (8): 27–32. https://doi.org/10.1093/jnci/djw322.

Soavelomandroso, A.P., F. Gaudin, S. Hoys, V. Nicolas, G. Vedantam, C. Janoir, and S. Bouttier. 2017. "Biofilm Structures in a Mono-Associated Mouse Model of Clostridium Difficile Infection." *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2017.02086.

Solomon, K. 2013. "The Host Immune Response to Clostridium Difficile Infection." *Therapeutic Advances in Infectious Disease* 1 (1): 19–35. https://doi.org/10.1177/2049936112472173.

Song, J.H., and Y.S. Kim. 2019. "Recurrent Clostridium Difficile Infection: Risk Factors, Treatment, and Prevention." *Gut and Liver* 13 (1): 16–24. https://doi.org/10.5009/gnl18071.

Sonnenburg, J.L. 2005. "Glycan Foraging in Vivo by an Intestine-Adapted Bacterial Symbiont." *Science* 307 (5717): 1955–59. https://doi.org/10.1126/science.1109051.

Sorg, J.A., and A.L. Sonenshein. 2008. "Bile Salts and Glycine as Cogerminants for Clostridium Difficile Spores." *Journal of Bacteriology* 190 (7): 2505–12. https://doi.org/10.1128/JB.01765-07.

Sorg, J.A., and A.L. 2009. "Chenodeoxycholate Is an Inhibitor of Clostridium Difficile Spore Germination." *Journal of Bacteriology* 191 (3): 1115–17. https://doi.org/10.1128/JB.01260-08.

Stevenson, E., N.P. Minton, and S.A. Kuehne. 2015. "The Role of Flagella in Clostridium Difficile Pathogenicity." *Trends in Microbiology* 23 (5): 275–82. https://doi.org/10.1016/j.tim.2015.01.004.

Sun, X., and S.A. Hirota. 2015. "The Roles of Host and Pathogen Factors and the Innate Immune Response in the Pathogenesis of Clostridium Difficile Infection." *Molecular Immunology* 63 (2): 193–202. https://doi.org/10.1016/j.molimm.2014.09.005.

Swidsinski, A., V. Loening-Baucke, and A. Herber. 2009. "Mucosal Flora in Crohn's Disease and Ulcerative Colitis - An Overview." *Journal of Physiology and Pharmacology* 60 (SUPPL.6): 61–71.

Swidsinski, A., J. Weber, V. Loening-Baucke, L.P. Hale, and H. Lochs. 2005. "Spatial Organization and Composition of the Mucosal Flora in Patients with Inflammatory Bowel Disease." *Journal of Clinical Microbiology* 43 (7): 3380–89. https://doi.org/10.1128/JCM.43.7.3380-3389.2005.

Tao, L., J. Zhang, P. Meraner, A. Tovaglieri, X. Wu, R. Gerhard, X. Zhang, *et al*. 2016. "Frizzled Proteins Are Colonic Epithelial Receptors for C. Difficile Toxin B." *Nature*.

https://doi.org/10.1038/nature19799.

Tasteyre, A., M.C. Barc, A. Collignon, H. Boureau, and T. Karjalainen. 2001. “Role of FliC and FliD Flagellar Proteins of Clostridium Difficile in Adherence and Gut Colonization.” *Infection and Immunity* 69 (12): 7937–40. https://doi.org/10.1128/IAI.69.12.7937-7940.2001.

Tasteyre, A., M.C. Barc, T. Karjalainen, P. Dodson, S. Hyde, P. Bourlioux, and P. Borriello. 2000. “A Clostridium Difficile Gene Encoding Flagellin.” *Microbiology* 146 (4): 957–66. https://doi.org/10.1099/00221287-146-4-957.

Temple, M.D., G.G. Perrone, and I.W. Dawes. 2005. “Complex Cellular Responses to Reactive Oxygen Species.” *Trends in Cell Biology* 15 (6): 319–26. https://doi.org/10.1016/j.tcb.2005.04.003.

The Integrative HMP (iHMP) Research Network Consortium. 2016. “The Integrative Human Microbiome Project: Dynamic Analysis of Cell Host Microbe Cell Host Microbe.” *Cell Host & Microbe* 16 (3): 276–89. https://doi.org/10.1016/j.chom.2014.08.014.

Thelen, T.D., R.M. Green, and S.F. Ziegler. 2016. “Acute Blockade of IL-25 in a Colitis Associated Colon Cancer Model Leads to Increased Tumor Burden.” *Scientific Reports* 6. https://doi.org/10.1038/srep25643.

Theriot, C.M., M.J. Koenigsknecht, P.E. Carlson, G.E. Hatton, A.M. Nelson, B. Li, G.B. Huffnagle, J.Z. Li, and V.B. Young. 2014. “Antibiotic-Induced Shifts in the Mouse Gut Microbiome and Metabolome Increase Susceptibility to Clostridium Difficile Infection.” *Nature Communications* 5. https://doi.org/10.1038/ncomms4114.

Theriot, C.M., C.C. Koumpouras, P.E. Carlson, I.I. Bergin, D.M. Aronoff, and V.B. Young. 2011. “Cefoperazone-Treated Mice as an Experimental Platform to Assess Differential Virulence of Clostridium Difficile Strains.” *Gut Microbes* 2 (6): 326–34. https://doi.org/10.4161/gmic.19142.

Thiele Orberg, E., H. Fan, A.J. Tam, C.M. Dejea, C.E. Destefano Shields, S. Wu, L. Chung, *et al*. 2017. “The Myeloid Immune Signature of Enterotoxigenic Bacteroides Fragilis-Induced Murine Colon Tumorigenesis.” *Mucosal Immunology* 10 (2): 421–33. https://doi.org/10.1038/mi.2016.53.

Tomkovich, S., C.M. Dejea, K. Winglee, J.L. Drewes, L. Chung, F. Housseau, J.L. Pope, *et al*. 2019. “Human Colon Mucosal Biofilms from Healthy or Colon Cancer Hosts Are Carcinogenic.” *Journal of Clinical Investigation* 129 (4): 1699–1712. https://doi.org/10.1172/JCI124196.

Tomkovich, S., Y. Yang, K. Winglee, J. Gauthier, M. Mühlbauer, X. Sun, M. Mohamadzadeh, *et al*. 2017. “Locoregional Effects of Microbiota in a Preclinical Model of Colon Carcinogenesis.” *Cancer Research* 77 (10): 2620–32. https://doi.org/10.1158/0008-5472.CAN-16-3472.

Vedantam, G., A. Clark, M. Chu, R. McQuade, M. Mallozzi, and V.K. Viswanathan. 2012. “Clostridium Difficile Infection: Toxins and Non-Toxin Virulence Factors, and Their Contributions to Disease Establishment and Host Response.” *Gut Microbes* 3 (2): 121–34. https://doi.org/10.4161/gmic.19399.

Velcich, A., W.C. Yang, J. Heyer, A. Fragale, C. Nicholas, S. Viani, R. Kucherlapati, *et al*. 2002. “Colorectal Cancer in Mice Genetically Deficient in the Mucin Muc2.” *Science*. https://doi.org/10.1126/science.1069094.

Viswanathan, V.K., M.J. Mallozzi, and G. Vedantam. 2010. “Clostridium Difficile Infection an Overview of the Disease and Its Pathogenesis, Epidemiology and Interventions.” *Gut Microbes* 1 (4): 234–42. https://doi.org/10.4161/gmic.1.4.12706.

Voelker, R. 2010. “Increased Clostridium Difficile Virulence Demands New Treatment Approach.” *JAMA - Journal of the American Medical Association* 303 (20): 2017–19. https://doi.org/10.1001/jama.2010.647.

Vogtmann, E., and J.J. Goedert. 2016. “Epidemiologic Studies of the Human Microbiome and Cancer.” *British Journal of Cancer* 114 (3): 237–42. https://doi.org/10.1038/bjc.2015.465.

Walk, S.T., D. Micic, R. Jain, E.S. Lo, I. Trivedi, E.W. Liu, L.M. Almassalha, *et al*. 2012. “Clostridium Difficile Ribotype Does Not Predict Severe Infection.” *Clinical Infectious Diseases* 55 (12): 1661–68. https://doi.org/10.1093/cid/cis786.

Warny, M., J. Pepin, A. Fang, G. Killgore, A. Thompson, J. Brazier, E. Frost, and L.C. McDonald. 2005. “Toxin Production by an Emerging Strain of Clostridium Difficile Associated with Outbreaks of Severe Disease in North America and Europe.” *Lancet*. https://doi.org/10.1016/S0140-6736(05)67420-X.

Welch, J.L.M., B.J. Rossetti, C.W. Rieken, F.E. Dewhirst, and G.G. Borisy. 2016. “Biogeography of a Human Oral Microbiome at the Micron Scale.” *Proceedings of the National Academy of Sciences of the United States of America* 113 (6): E791–800. https://doi.org/10.1073/pnas.1522149113.

Wilson, K.H. 1983. “Efficiency of Various Bile Salt Preparations for Stimulation of Clostridium Difficile Spore Germination.” *Journal of Clinical Microbiology* 18 (4): 1017–19. https://doi.org/10.1128/jcm.18.4.1017-1019.1983.

Wohlan, K., S. Goy, A. Olling, S. Srivaratharajan, H. Tatge, H. Genth, and R. Gerhard. 2014. “Pyknotic Cell Death Induced by Clostridium Difficile TcdB: Chromatin Condensation and Nuclear Blister Are Induced Independently of the Glucosyltransferase Activity.” *Cellular Microbiology* 16 (11): 1678–92. https://doi.org/10.1111/cmi.12317.

Wong, S.H., L. Zhao, X. Zhang, G. Nakatsu, J. Han, W. Xu, X. Xiao, *et al*. 2017. “Gavage of Fecal Samples From Patients With Colorectal Cancer Promotes Intestinal Carcinogenesis in Germ-Free and Conventional Mice.” *Gastroenterology* 153 (6): 1621-1633.e6. https://doi.org/10.1053/j.gastro.2017.08.022.

Wu, G.D., J. Chen, C. Hoffmann, K. Bittinger, Y. Chen, S.A. Keilbaugh, M. Bewtra, *et al*. 2011. “Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes.” *Science* 334 (October): 105–9. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3368382&tool=pmcentrez&rendertype=abstract.

Wu, S., K.-J.J. Rhee, E. Albesiano, S. Rabizadeh, X. Wu, H.-R.R. Yen, D.L. Huso, *et al*. 2009. “A Human Colonic Commensal Promotes Colon Tumorigenesis via Activation of T Helper Type

17 T Cell Responses.” *Nature Medicine* 15 (9): 1016–22. https://doi.org/10.1038/nm.2015.

Yang, Y., W. Weng, J. Peng, L. Hong, L. Yang, Y. Toiyama, R. Gao, *et al*. 2017. “Fusobacterium Nucleatum Increases Proliferation of Colorectal Cancer Cells and Tumor Development in Mice by Activating Toll-Like Receptor 4 Signaling to Nuclear Factor−κB, and Up-Regulating Expression of MicroRNA-21.” *Gastroenterology*. https://doi.org/10.1053/j.gastro.2016.11.018.

Zackular, J.P., M.A.M. Rogers, M.T. Ruffin, and P.D. Schloss. 2014. “The Human Gut Microbiome as a Screening Tool for Colorectal Cancer.” *Cancer Prevention Research* 7 (11): 1112–21. https://doi.org/10.1158/1940-6207.CAPR-14-0129.

Zheng, Y., Y. Luo, Y. Lv, C. Huang, Q. Sheng, P. Zhao, J. Ye, *et al*. 2017. “<I>Clostridium Difficile</I> Colonization in Preoperative Colorectal Cancer Patients.” *Oncotarget*. https://doi.org/10.18632/oncotarget.14424.

Zhu, D., J.A. Sorg, and X. Sun. 2018. “Clostridioides Difficile Biology: Sporulation, Germination, and Corresponding Therapies for C. Difficile Infection.” *Frontiers in Cellular and Infection Microbiology* 8 (FEB): 1–10. https://doi.org/10.3389/fcimb.2018.00029.

Curriculum Vitae

Jie Chen
Email: jchen212@jhmi.edu

EDUCATION
PhD in Molecular Microbiology and Immunology, Sep. 2015- Jun. 2021
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

Bachelor of Medicine and Master of Medicine (M.D equivalent), Sep.2003- June. 2010
School of Medicine, Nankai University, Tianjin, China

TEACHING EXPERIENCE
- Teaching Assistant, Jul. 2018- Aug. 2018
  Course: Techniques in Molecular Biology, Summer Mini-Term III. Johns Hopkins Department of Biology
- Teaching Assistant, Jan. 2018- Mar. 2018
  Course: Pathogenesis of Bacterial Infections, Johns Hopkins Bloomberg School of Public Health
- Teaching Assistant, Jan. 2017 & Jan. 2018
  Course: Techniques in Molecular Biology, Johns Hopkins Bloomberg School of Public Health

WORK EXPERIENCE
- Research Associate, Dec. 2013- May 2015
  Flavell Lab, Department of Immunobiology, Yale School of Medicine, New Haven, CT, USA
- Resident, Oct. 2010- Nov. 2013
  Institute of Geriatric Cardiology, Chinese PLA General Hospital, Beijing, China
- Intern, Aug. 2008-Jun. 2010
  Chinese PLA General Hospital, Beijing, China

RESEARCH EXPERIENCE
PhD thesis research, April. 2016- present
Advisor: Cynthia L. Sears, M.D
Department of Medicine. Department of Oncology and the Sidney Kimmel Comprehensive Cancer Center
Johns Hopkins School of Medicine, Baltimore, MD 21287, USA
• Using $Apc^{Min/+}$ mouse model to test if *Clostridioides difficile* contributes to colon tumorigenesis.
• Mucus degradation and bacterial mucosal adherence modulate microbial virulence and capacity to induce colon tumorigenesis.

Research Associate, Dec. 2013- May. 2015
Advisor: Richard A. Flavell, Ph.D., FRS
Department of Immunobiology, Yale School of Medicine, New Haven, CT 06520-8011, USA
- Development of a mouse model to support human erythropoiesis for the studies on blood stage of malaria infection.

Research Assistant, Jun. 2009-Jun. 2012
Advisor: Yu Wang, M.D and Lian-Ru Gao, M.D
Institute of Geriatric Cardiology, Chinese PLA General Hospital, Beijing, China
- The randomized multicenter clinical trial: the effects of mesenchymal stem cells from Wharton's Jelly of human umbilical cord on heart function of patients with acute myocardial infarction (State High-Tech Development Plan).

PUBLICATIONS

- Song Y, Shan L, Gbyli R, Liu W, Strowig T, Patel A, Fu X, Wang X, Xu ML, Gao Y, Qin A, Bruscia EM, Tebaldi T, Biancon G, Mamillapalli P, Urbonas D, Eynon E, Gonzalez DG, **Chen J**, Krause DS, Alderman J, Halene S, Flavell RA. Combined liver-cytokine humanization comes to the rescue of circulating human red blood cells. Science. 2021, 371(6533):1019-1025.
- Nilaratanakul V, **Chen J**, Tran O, Baxter VK, Troisi EM, Yeh JX, Griffin DE. Germ Line IgM Is Sufficient, but Not Required, for Antibody-Mediated Alphavirus Clearance from the Central Nervous System. J Virol. 2018, 14: 92-97.
- Chung L, Orberg ET, Geis AL, Chan JL, Fu K, DeStefano Shields CE, Dejea CM, Fathi P, **Chen J**, Finard BB, Tam AJ, McAllister F, Fan H, Wu X, Ganguly S, Lebid A, Metz P, Van Meerbeke SW, Huso DL, Wick EC, Pardoll DM, Wan F, Wu S, Sears CL, Housseau F. Bacteroides fragilis Toxin Coordinates a Pro-carcinogenic Inflammatory Cascade via Targeting of Colonic Epithelial Cells. Cell Host Microbe. 2018, 23: 203-214.
- Herndler-Brandstetter D, Shan L, Yao Y, Stecher C, Plajer V, Lietzenmayer M, Strowig T, de Zoete MR, Palm NW, **Chen J**, Blish CA, Frleta D, Gurer C, Macdonald LE, Murphy AJ, Yancopoulos GD, Montgomery RR, Flavell RA. Humanized mouse model supports development, function, and tissue residency of human natural killer cells. Proc Natl Acad Sci U S A. 2017 Nov 7;114(45): E9626-E9634.
- **Chen J**, Dominque J, and Sears CL. Dysbiosis of Microbiota in Human Cancers: Evidence of Association and Causality. Semin Immunol. 2017, 32: 25-34.
- Zhou S, **Chen J**, Xu RY and Wu HY. Factors associated with the use of percutaneous coronary intervention in elderly Chinese patients with a first ST elevated acute myocardial infarction. Patient Prefer. Adher. 2014, 8: 257–262.
- **Chen J**, Xue Q, Bai J, Gao L, Tian JW, Li K, Xu Q, Li YH, Wang Y. Incomplete revascularization in the drug eluting stents era permits meaningful long-term (12-78 months) outcomes in patients ≥75 years with acute coronary syndrome. J Geriatr Cardiol. 2012, 9: 336−343.

CONFERENCE ABSTRACTS

- Chen J, Wu S, McMann M, Lansiquot C, Wu X, James White, Markham NO, Besharati S, Anders R, Tomkovich S, Jobin C, and Sears CL. *Clostridioides difficile* colonization induces colon tumorigenesis in a murine model. *2020 Conference of Anaerobe Society of the Americas (July 2020, Zoom)*
- Chen J, Wu S, Drewes JL, Domingue JC, Chan J, Allen J, Wu XQ, Fleckenstein JM, Sears CL. Mucus-degrading Bacteria Modulate Mucosal Adherence of Genotoxic Bacteria for Promoting Colon Tumorigenesis. *2018 Conference of Anaerobe Society of the Americas (July 2018, Las Vegas, NV)*
- Shan L, **Chen J**, Strowig T, Rongvaux A, Garg A, Mamoun CB, Flavell R. Development of a mouse model for human blood stage malaria infection. *2014 Immunobiology Retreat of Yale University (October 2014, Southbury, CT)*.
- Martinez-Morilla S, Shan L, **Chen J**, Flavell R, Hattangadi S. Using the humanized mouse model to help understand the role of the macrophage during terminal human erythropoiesis. *Red Blood Cell Meeting (Fall 2014, Toronto)*.
- **Chen J**, Yang FF, Bai J, Zhang GM, Zhang LW, Wang Y. The Effect of Syntax Score on the In-hosptial Efficacy and Safety of Percutaneous Coronary Intervention in the Aged Patients. *China Interventional Therapeutics Conference (April 2010, Beijing)*.

HONORS AND AWARDS

- First Prize in the Anaerobe 2018 Young Investigator's Award Competition; Student Travel Grant for *2018 Conference of Anaerobe Society of the Americas* (2018)
- 2015-2016, 2017-2018, 2018-2019 and 2019-2020 Wittler Student Scholarship