Targeting the Toxin B glucosyltransferase domain of NAP1/B1/027 *Clostridioides difficile* using an attenuated *Salmonella enterica* Typhimurium vaccine vector

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December 2022

A thesis submitted to the Faculty of Graduate Studies and Research of McGill University in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Clostridioides difficile (*C. difficile*) is the most common cause of nosocomial antibiotic-associated diarrhea in developed countries and is classified as an urgent threat by the Centers of Disease Control and Prevention (CDC). Most strains of *C. difficile* release toxins A (TcdA) and B (TcdB) that mediate disease. Annually, there are approximately 450,000 new cases and 29,000 deaths in the USA alone resulting in an economic burden of $5.4 billion dollar. The emergence of hypervirulent strains, notably NAP1/B1/027, in the 2000s and the relative inefficiency of current treatments against these strains highlight the need for a vaccine. Therefore, we proposed a vaccine consisting of attenuated *Salmonella enterica* serovar Typhimurium (*S.* Tm) strains YS1646 that express the immunogenic portions of TcdB (i.e. the glucosyltransferase domain (GTD) and the receptor binding domain (RBD)). Since GTD is highly conserved across many *C. difficile* strains, we hypothesized that this vaccine would protect mice from lethal challenge with NAP1/B1/027 strains and possibly contribute to protection against all strains. We aimed to modify a murine model for NAP1/B1/027 challenge and to evaluate the protective efficacy against NAP1/B1/027 challenge of a GTD-expressing YS1646 vaccine compared to a more traditional vaccine targeting the RBD of *C. difficile* TcdB (rbdB). The optimal dose for infection with a NAP1/B1/027 clinical isolate was demonstrated to range between $4.07x10^4$ and $9.83x10^4$ colony forming units (CFU)/ mouse. The GTD was confirmed to be immunogenic in C57BL/6 mice when three intramuscular (i.m.) doses of rGTD (10µg alum-adjuvanted) were administered on days 0, 21 and 35 (serum anti-TcdB IgG titers $>1.3x10^4$ ng/mL). Female C57BL/6 mice that were vaccinated with the traditional rbdB multimodal vaccine (a single i.m. dose of alum-adjuvanted recombinant rbdB (rrbdB) on D0 and three p.o. doses of YS1646-vectored rbdB on D0, D2, D4) yielded an efficacy of 80% against lethal challenge with a NAP1/B1/027 strain. The 'solo' GTD multimodal (three i.m. doses of alum-adjuvanted rGTD on D0, D21, D35; and three p.o. doses of YS1646-vectored GTD on D35, D37, D39) and the combined GTD + rbdB multimodal (three i.m. doses of rGTD on D0, D21, D35; a single i.m. dose of rrbdB on D35; and three p.o. doses of each antigen in a YS1646 vector on D35, D37, D39) vaccines yielded an efficacy of 100%. Interestingly, TcdB-specific IgG titers were higher in the combined GTD + rbdB vaccine group compared to the 'solo' GTD vaccine group (3.15×10^4 ng/mL and 7.3×10^3 ng/mL, respectively). While the combined GTD + rbdB multimodal vaccine seemed to be the most promising construct, an evaluation of the mucosal response is required.

RÉSUMÉ

Clostridioides difficile (*C. difficile*) est la cause la plus fréquente de diarrhée nosocomiale associée aux antibiotiques dans les pays développés et est classé comme une menace urgente par les Centres pour le contrôle et la prévention des maladies (CDC). La plupart des souches de *C. difficile* libèrent des toxines A (TcdA) et B (TcdB) qui médient la maladie. Chaque année, il y a environ 450 000 nouveaux cas et 29 000 décès aux États-Unis seulement, ce qui représente un fardeau économique de 5,4 milliards de dollars. L'émergence de souches hypervirulentes, notamment NAP1/B1/027, dans les années 2000 et la relative inefficacité des traitements actuels contre ces souches mettent en évidence la nécessité d'un vaccin. Par conséquent, nous avons proposé un vaccin composé de souches atténuées de *Salmonella enterica* sérovar Typhimurium (S. Tm) YS1646 qui expriment les parties immunogènes de TcdB (le domaine de la glucosyltransférase (GTD) et le domaine de liaison au récepteur (RBD)). Étant donné que GTD est hautement conservé dans de nombreuses souches de *C. difficile*, nous avons émis l'hypothèse que ce vaccin protégerait les souris d'une provocation mortelle avec une souche NAP1/B1/027 et contribuerait peut-être à la protection contre toutes les souches. Nous avons cherché à modifier un modèle murin pour la provocation avec NAP1/B1/027 et à évaluer l'efficacité protectrice contre la provocation avec NAP1/B1/027 d'un vaccin YS1646 exprimant GTD par rapport à un vaccin plus traditionnel ciblant le RBD de *C. difficile* TcdB (rbdB). Nous avons démontré que la dose optimale pour l'infection par un isolat clinique NAP1/B1/027 était comprise entre $4,07x10^4$ et $9,83x10^4$ unités formant colonies (UFC)/souris. La GTD s'est avérée immunogène chez les souris C57BL/6 lorsque trois doses intramusculaires (i.m.) de rGTD (10 µg avec adjuvant d'aluminium) ont été administrées aux jours 0, 21 et 35 (titres sériques d'IgG anti-TcdB > 1,3 x 10^4 ng/mL). La vaccination multimodale des souris C57BL/6 avec rbdB (une seule dose i.m. de 3µg rbdB recombinant [rrbdB] avec adjuvant d'aluminium et trois doses oral [p.o.] de rbdB exprimé par le vecteur YS1646) a donné une efficacité de 80% contre la provocation mortelle avec la souche NAP1/B1/027 utilisée. Les vaccins multimodals de GTD «solo» (trois doses i.m. de rGTD avec adjuvant alun à J0, J21, J35; et trois doses p.o. de GTD exprimé par un vecteur YS1646 à J35, J37, J39) et GTD + rbdB combiné (trois doses i.m. de rGTD à J0, J21, J35; une seule dose i.m. de rrbdB à J35; et trois doses p.o. de chaque antigène exprimé par le vecteur YS1646 à J35, J37, J39) ont donné une efficacité de 100%. Il est intéressant de noter que les titres d'IgG spécifiques au TcdB étaient plus élevés dans le groupe de vaccin GTD + rbdB combiné par rapport au groupe de vaccin GTD «solo» (3,15 x 10^4 ng/mL et

7,3 x 10^3 ng/mL, respectivement). Alors que le vaccin multimodal GTD + rbdB combiné semblait être la construction la plus prometteuse, une évaluation de la réponse muqueuse est nécessaire.

ACKNOWLEDGEMENTS

First, I want to thank my supervisor, Dr. Brian J. Ward, for giving me the opportunity of doing a Masters in their lab and their endless support throughout my degree. To Brian, thank you so much for your mentorship, expertise, wisdom, and words of encouragement. I've learned so much from you like maintaining a disciplined yet adventurous life (as I can tell from the funny stories you tell us; please be careful when you go kayaking next time). You also encouraged me to always seek knowledge whether it be related to your field or not. You inspire me to become a better version of myself, not just as a scientist, but as an individual as well. I just hope that I'd also be able to have an exciting and adventure-filled life like yours that I can look back to and feel proud. Thank you Dr. Samantha Gruenheid and Dr. Todd Lee for accepting to be on my committee despite your hectic schedules, and for your time and advice. Acknowledgement to McGill University, Research Institute of the McGill University Health Center and Aviex for funding.

A huge thank you to Dr. Kaitlin Winter for taking me under your wing when I was a lost newcomer and sharing my struggles throughout my time in the lab (let's go team *C. diff*!). Thank you for answering every single one of my questions. I will never forget when you consoled me by suggesting that I change our counter back to zero for the "number of days spent without crying about science" after a failed experiment to make me laugh, thank you. Thank you to Annie Beauchamps for your immense help when working with mice (and sorry for the crazy number of mice per experiment). Thank you, Georgia Stavrakis, for all your work on this project before I took over. Thank you, Angela and Louis obviously. A huge shout-out to Iris and Selena for making lab work fun; to Dr. Adam Hassan (my T.A. tuned lab buddy) for referring me to Brian; Fatima and Raidan for funny evening talks and guidance; and to all Ward/Ndao members.

Thank you to my first mentor, Dr. Inés Colmegna and everyone in the Colmegna lab: Peter Jeon, Maximilien Lora and Natalia França.

I want to thank my family: Mother India (Kinjal Joshi) and Father India (Vidyut Upadhyaya), Gondu (Keisha Upadhyaya) and bun-kids Milo and Milan, including my best friends Diksha and Kenya, who have supported and comforted me, laughed and cried with me and loved me whole-heartedly. Thank you for your encouragement and your strength! Thank you, Mota Dada (Kanchanlal C. Upadhyaya), for being the reason why I keep going. I hope you're proud. Rest in Peace. Love you all, forever and always!

Lastly, I want to acknowledge the mice who participated in the experiments. Rest in Peace.

PREFACE

This Master's thesis was written in accordance with McGill University's "Guidelines for Thesis Preparation". The candidate, Pavitra Upadhyaya (PU), has chosen to present their thesis in a traditional monograph format. All work towards this thesis was performed under the supervision of Dr. Brian J. Ward (BW). The candidate is the first author of the thesis.

Author contributions are as followed: Overall, the project was designed and envisioned by BW. The development of *Salmonella enterica* serovar Typhimurium (S. Tm) strain YS1646 expressing GTD and *Escherichia coli* expressing rGTD was done by Georgia Stavrakis (GS). The optimization of NAP1/B1/027 culture and enzyme-linked immunosorbent assays (ELISAs) were designed and performed by Pavitra Upadhyaya (PU) with the guidance of Dr. Kaitlin Winter (KW) and BW. *In vivo* experiments (NAP1/B1/027 murine model, rGTD immunogenicity and efficacy studies of candidate vaccines) were desgined and performed by PU with the guidance of KW and BW and with the help of Annie Beauchamps (AB). Statistical analyses were performed by PU. PU wrote this monograph with editorial guidance and direction from BW.

LIST OF ABBREVIATIONS

C. difficile	*Clostridioides difficile*
TcdA	Toxin A
TcdB	Toxin B
CDI	*C. difficile* infection
CDT	*C. difficile* transferase/ binary toxin
CDC	Centers of Disease Control and Prevention
U.S.	United States
CA	Community-associated
ADP	Adenosine diphosphate
NAP1/B1/027	Pulse-field gel electrophoresis type 1, restriction endonuclease analysis type B1, polymerase chain reaction ribotype 027
RBD	Receptor binding domain
GTD	Glucosyltransferase domain
PMC	Pseudomembranous colitis
rCDI	Recurrent CDI
PPIs	Proton pump inhibitors
H2RAs	H2 receptor antagonists
HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
EIAs	Enzyme immunoassays
IDSA	Infectious Diseases Society of America
SHEA	Society for Healthcare Epidemiology of America
GDH	Glutamate dehydrogenase
NAAT	Nucleic acid amplification test
FDA	Food and Drug Administration
FMT	Fecal microbiota transplant
i.p	Intraperitoneal
UV	Ultraviolet radiation

B. subtilis	*Bacillus subtilis*
DPA	Pyridine-2,6-dicarboxylic acid
mRNA	Messenger RNA
CaDPA	Calcium-chelated DPA
CdeC	Cysteine-rich exosporium morphogenetic protein
CcpA	Catabolite control protein A
GTP	Guanosine triphosphate
Spo0A	Stage 0 sporulation protein A
SHKs	Sensor histidine kinases
Csp	Cold-shock protein
PBAs	Primary bile acids
DCA	Deoxycholic acid
SCLEs	Spore cortex lytic enzymes
PaLoc	Pathogenicity locus
SCFAs	Short-chain fatty acids
CPD	Cysteine protease domain
CROPs	Combined repetitive peptides
gp	Glycoprotein
siRNA	Small interfering RNA
CSPG 4	Chondroitin sulfate proteoglycan 4
FZD	Wnt receptor Frizzled
$InsP_6$	Inositol hexakisphosphate
UDP	Uridine diphosphate
LSR	Lipolysis-stimulated lipoprotein receptor
HSP	Heat shock protein
ECM	Extracellular matrix
SLPs	Surface layer proteins
CWPs	Cell wall proteins

PS	Polysaccharide
HMW	High molecular weight
LMW	Low molecular weight
Ig	Immunoglobulin
c-di-GMP	Cyclic dimeric guanosine monophosphate
WT	Wild type
ILCs	Innate lymphoid cells
DCs	Dendritic cells
AMPs	Antimicrobial peptides
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
IECs	Intestinal epithelial cells
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
LPS	Lipopolysaccharide
PRRs	Pattern recognition receptors
TLRs	Toll-like receptors
iTregs	Inducible regulatory T cells
Th17	T helper 17 cells
IL	Interleukin
TGF-β	Transforming growth factor β
IgA	Immunoglobulin A
AP-1	Activator protein 1
MIP-2	Macrophage inflammatory protein 2
TNF-α	Tumor necrosis factor α
NK	Natural killer
IFN	Interferon
MIF	Macrophage migration inhibition factor

MIP-1α	Macrophage inflammatory protein 1α
rCDI	Recurrent CDI
NO	Nitric oxide
APCs	Antigen-presenting cells
MHC-II	Major histocompatibility complex class II
TSLP	Thymic stromal lymphopoietin
GM-CSF	Granulocyte-macrophage colony stimulating factor
KO	Knock-out
MBCs	Memory B cells
Tfh	T follicular helper cells
Th1	T helper 1 cells
GCs	Germinal centers
sIgA	Secretory IgA
NTCD	Non-toxigenic *C. difficile*
S. enterica	*Salmonella enterica*
S. Tm	*S. enterica* serovar Typhimurium
B. anthracis	*Bacillus anthracis*
PA	Protective antigen
E. coli	*Escherichia coli*
T3SS	Type 3 secretion system
SPI	*Salmonella* pathogenicity island
CFU	Colony forming units
rbdA	RBD of TcdA
rbdB	RBD of TcdB
rrbdA	Recombinant rbdA
rrbdB	Recombinant rbdB
i.m.	Intramuscular
p.o.	Oral/*per os*

PBS	Phosphate-buffered saline
ELISA	Enzyme-linked immunosorbent assay
pagC_SspH1_GTD	YS1646 strain expressing GTD
rGTD	Recombinant GTD
pET28b-GTD	pET28b plasmid encoding GTD DNA
pET28b-rbdB	pET28b plasmid encoding rbdB DNA
LB	Luria broth
BHIS	Brain heart infusion
IPTG	Isopropyl-β-D-thiogalactopyranoside
NTA	Nitrilotriacetic acid
His	Histidine
BSA	Bovine serum albumin
OD	Optical density
BCA	Pierce Bicinchoninic Acid
RI-MUHC	McGill University Health Center Research Institute
ARD	Animal Resource Division
Alum	Aluminum hydroxide gel
SspH2_sspH2_rbdB	YS1646 strain expressing rbdB
PI	Protease inhibitor
HRP	Horse-radish peroxidase
TMB	3,3',5,5'-tetramethylbenzidine
R^2	R squared
CO_2	Carbon dioxide
GMP	Good manufacturing product
PBMCs	Peripheral blood mononuclear cells

CHAPTER 1: LITERATURE REVIEW

1.1 History of *Clostridioides difficile*

Clostridioides difficile (*C. difficile*) is a strictly anaerobic, spore-forming, Gram-positive gastrointestinal pathogen that may or may not produce toxins A (TcdA), B (TcdB) and binary toxin (CDT) (also known as *C. difficile* transferase) depending on the strain (1). Some strains produce only one toxin (TcdA or TcdB) and some strains do not to produce any toxin (2, 3). Strains that produce CDT are often associated with hypervirulence (4). In this thesis, the illness caused by this pathogen will be referred to as *C. difficile* infection (CDI) (1).

The first description of *C. difficile* was published in 1935 by Hall and O'Toole who analyzed stool of newborn healthy infants for microbial changes (5). The bacterium was described as a spore-forming, rod-shaped strict anaerobe, and was named *Bacillus difficilis* due to the difficulty of isolating this bacterium (5). Further studies on this bacterium revealed that some strains were capable of producing a thermo-labile toxin (inactivated in 5 minutes at 60˚C) that could be neutralized by an antiserum given to guinea pigs up to 4 hours after the toxin was given subcutaneously (6). In 1938, *Bacillus difficilis* was reclassified as *Clostridium difficile* and in 2016, *Clostridium difficile* was renamed *Clostridioides difficile* (7, 8).

1.2 Epidemiology of *Clostridioides difficile*

C. difficile is the leading cause of nosocomial diarrhea in North America and Europe representing 10%-20% of cases and was recently classified as an urgent public threat by the Centers of Disease Control and Prevention (CDC) (9). The incidence of CDI in North America and Europe has increased two- to four-fold over the past decade with approximately 453 000 annual cases and 29,000 deaths within 30 days of diagnosis resulting in an economic burden of approximately $5.4 billion in the United States (U.S.) alone (10-12). In Europe, the annual economic burden is estimated to be approximately EU €3000 million (~€5000- €15,000/case) (13). In 2012, Canada reported 37 690 episodes of CDI of which 10 900 (27%) were recurrences, representing an estimated annual cost to society of $281 million (14).

Although, CDI has historically been considered a hospital-acquired disease, recent data demonstrate an alarming increase in cases of community-associated (CA)-CDI (15). In the U.S., approximately one third of cases are CA-CDI and symptom onset is highly correlated with

antibiotic use (16). Several studies have discovered *C. difficile* spores in the environment (soil, water, and food) and in animals (pets and farm), thus contributing to *C. difficile* transmission (17-41). For example, a study in 17 supermarkets of Saudi Arabia showed a prevalence of 0.75% in retail baskets and trolleys (17). Another study demonstrated a prevalence of 87.5% in river waters of South Wales (18).Therefore, *C. difficile* is a ubiquitous bacterium found in the environment that can persist on inanimate surfaces for months by forming resistant spores (42-44).

1.3 Emergence of hypervirulent strains (NAP1/B1/027)

Between 1991 and 2003, Pépin et al. observed an unprecedented increase in the incidence and disease severity of CDI in the Centre hospitalier universitaire de Sherbrooke in Canada (45, 46). In 2004, a study by Loo et al. showed that 84.1% of these cases were caused by a variant of *C. difficile* that was highly resistant to fluoroquinolones, possessed a mutation in the negative regulator of toxin synthesis, *tcdC*, and expressed an additional toxin, an adenosine diphosphate (ADP)-ribosyltransferase called CDT (47). This variant was characterized using pulsed-field gel electrophoresis (type 1), restriction endonuclease analysis (type B1), polymerase chain reaction (ribotype 027) – hence NAP1/B1/027 (47). Presently, between 22%-36% of all CDI cases in North America are caused by NAP1/B1/027 strains (48). NAP1/B1/027 remains the predominant circulating hypervirulent strain and continues to be associated with high mortality rates (49). Further studies demonstrated its ability to hypersporulate, overexpress toxins A and B, and resist several antibiotics (e.g. rifampicin, clindamycin, imipenem and chloramphenicol) (50-53). Recently, Lanis and colleagues reported high DNA sequence variation between historical strains and NAP1/B1/027 strains primarily in the C-terminus of TcdB or the receptor binding domain (RBD) (54, 55) such that antibodies mounted against the RBD of TcdB from historical strains are unable to neutralize TcdB from NAP/B1/027 (54).

This project focuses on the conserved and immunogenic N-terminal region of TcdB, the glucosyltransferase domain (GTD) (56, 57).

1.4 Infectious cycle and clinical presentation of *Clostridioides difficile* infection

CDI most often begins with ingestion of spores via the fecal-oral route (58). An imbalance of the intestinal microbiota (i.e. dysbiosis) due to broad-spectrum antibiotics treatment (e.g. clindamycin, cephalosporins and penicillins) results in decrease in colonization resistance, and

subsequently promotes establishment of *C. difficile* (59, 60). In the intestinal lumen under appropriate conditions, *C. difficile* spores germinate and start to release one or more toxins (e.g. TcdA, TcdB, CDT) which mediate *C. difficile* disease (1). Non-toxigenic C. difficile strains do not cause CDI (1, 61).

The clinical presentation of CDI varies from asymptomatic carriage (4-15% of healthy population) to mild-to-severe diarrhea (62-64). If left untreated, symptomatic patients may progress to develop pseudomembranous colitis (PMC), toxic megacolon, intestinal perforation, sepsis, and multi-organ failure (64). The mortality rate directly attributable to CDI ranges from 6.9% in patients aged 18-64 years to 19.7% for patients aged 85 years or older (49).

1.5 Risk factors of *Clostridioides difficile* infection

The main risk factors for developing symptomatic CDI include the use of pharmacological agents (i.e. antibiotics and gastric acid suppressors), compromised immunity, and advanced age (≥65 years) (65). Clindamycin, cephalosporins and fluoroquinolones, are associated with the greatest risk of CDI and recurrent CDI (rCDI) in healthcare settings or the community (66-71). It has been hypothesized that the innapropriate use of fluoroquinolones promulgated NAP1/B1/027 strains (72). Other CDI risk factors include the use of proton pump inhibitors (PPIs) and H2 receptor antagonists (H2RAs) (73, 74). The continued use of PPIs but not H2RAs, is also associated with the risk of rCDI (73, 74).

Some immunocompromised individuals that are unable to mount an effective immune response against *C. difficile* are also at increased risk of CDI (75). A limited number of studies have investigated the incidence of CDI in the hematology-oncology population (6%-31% vs 1% in the general patient population) (76-78), patients with solid organ tumors (17.3%) (79), solid organ transplant recipients (up to 23% in patients with a lung transplant) and children with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) (17%-24%) (80-82). Some immunocompromised patients are also at higher risk of rCDI (83).

The association between increased age and the risk of CDI is well-known and is explained by frequent healthcare exposure, compromised immunity, and increased use of antibiotics (84). Mortality rates in the elderly population increases from 5% in 61-70 year-olds to >10% in patients aged 80 or over (85). In 2018, approximately 86% of all deaths caused by CDI occurred in

Americans aged >65 years placing this illness as the 19th leading cause of death in the U.S. for people in this age group (86).

1.6 Diagnosis and treatment

A number of approaches are used in the diagnosis of CDI including enzyme immunoassays (EIAs), cellular cytotoxicity assays, anaerobic culture and/or DNA-based tests (11, 65). Additionally, imaging techniques such as abdominal x-rays, ultrasound and computed tomography or a colonoscopy are also used to evaluate CDI symptoms and determine disease severity (65). Currently, cytotoxicity assays and stool culture are less frequently used due to the longer response time (≥48 hours) and the need for anaerobic conditions that are not commonly available (11).

CDI is defined by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA) as having 1) ≥ three unformed stools in 24 hours, 2) a positive stool toxin test, and 3) a positive result in the glutamate dehydrogenase (GDH) test and/or nucleic acid amplification test (NAAT) (87). The GDH test is an EIA used to identify the presence of GDH, a cell wall protein produced by *C. difficile* (88). It has 85-95% sensitivity and 89-99% specificity and a response time of 15-45 minutes, but it cannot differentiate toxigenic strains from non-toxigenic strains which is why this test is followed by an EIA identifying the presence of TcdA, or TcdA and TcdB (48-96% sensitivity, 75-99% specificity) (65). Among the NAAT, there are two common methods: polymerase chain reaction (PCR) for the identification of *tcdB* (sensitivity 84-96%, specificity 96-99%) and loop-mediated isothermal amplification for the identification of *tcdA* (sensitivity 92-96%, specificity 98%); each with a theoretical response time of 45-180 minutes (65). A NAAT test followed by an EIA test (i.e. multi-step algorithm) is currently the most accurate and rapid way to establish a CDI diagnosis (89).

Ironically, antibiotics remain the mainstay for CDI treatment. Broad-spectrum antibiotics such as metronidazole and vancomycin are used to treat mild, or severe and recurrent CDI, respectively (90). In 2011, the Food and Drug Administration (FDA) approved a narrow-spectrum antibiotic, fidaxomicin, that demonstrates higher efficiency than vancomycin in reducing the risk of recurrence (91). Even with optimal treatment however, recurrence rates remain stubbornly between 15% and 35%, reflecting their inefficiency of treating this antibiotic-induced disease with more antibiotics (92). To address this issue and given the protective role of a healthy gut microflora in controlling CDI, several groups have investigated the potential benefits of fecal microbiota

transplant (FMT) which focusses on restoring the gut microbiota to increase resistance (93). Although FMT has demonstrated great promise, this rather cumbersome approach remains an experimental therapy due to inconsistent efficiency and unknown long-term side-effects (93). Another strategy to minimize recurrence includes passive immunization. Bezlotoxumab, a monoclonal anti-TcdB antibody, was FDA-approved in 2016 for the prevention of recurrence (94, 95). Although bezlotoxumab can reduce the risk of recurrence by ~10%, the high cost limits its use (94-96). These observations strongly support the development for preventative strategies such as vaccines.

1.7 Animal models

The first well-documented case of PMC was reported in 1893 by J.M. Finney and William Osler (97). They mentioned diphteritic membranes in an autopsy of a 22-year old woman who had died with post-operative diarrhea (97). PMC was described as a post-operative complication that can only be diagnosed after death (97). At first, *Staphylococcus aureus* was thought to be the causative agent of PMC (98). It took 40 years to recognize *C. difficile* as the most important etiological agent of antibiotic-associated PMC (99-102). This was possible due to the identification of a good animal model, the hamster. In 1978, Bartlett et al. demonstrated that stool samples from PMC patients caused enterocolitis in hamsters and isolated *C. difficile* in the feces of these animals (98, 101, 102). Since then, the hamster model has been widely used to study CDI, *C. difficile* virulence and toxins, and to test potential treatments (103). The hamster model is still the most widely used animal model of CDI despite important limitations: the wide spectrum of CDI symptoms in humans cannot be replicated in hamsters, and the lack of hamster-specific reagents and genetically modified animals (103). Novel animal models were therefore vital to expand our understanding of CDI.

In 2008, Chen et al. described the first murine model of CDI (104). Because mice are not intrinsically susceptible to *C. difficile* infection, Chen's team administered an antibiotic cocktail (kanamycin, gentamicin, colistin, metronidazole and vancomycin) in drinking water for 3 days, in addition to an intraperitoneal (i.p.) dose of clindamycin 1 day prior to challenge with different doses of *C. difficile* (104). The rationale was to induce dysbiosis of the murine gut microbiome thus rendering the mice susceptible to CDI (104). Infected mice developed diarrhea and lost weight. Additionally, typical human-like features in histopathological analyses of colonic tissues

were observed (104). An advantage of this model is that disease severity was dose- and strain-dependent, thus the range of CDI symptoms seen in humans could be better replicated in mice (104).

Winter et al. from our laboratory has developed a mouse model similar to that described by Chen et al. to test classical RBD-targeting vaccine candidates against a laboratory/historical strain of *C. difficile* (VPI 10463) (105). The current project involved the modification of Winter's model for the study of NAP1/B1/027 strains.

1.8 Pathogenesis of *Clostridioides difficile*

1.8.1 Sporulation and germination

C. difficile is a spore-forming bacterium (1). The production of oxygen-, heat-, and ultraviolet radiation (UV)-resistant spores that survive disinfection with common ethanol-based products allows for persistence and effective transmission of the pathogen via the fecal-oral route (106-108). Much of what is known about sporulation in *C. difficile* relies on what is known about *Bacillus subtilis* (*B. subtilis*) sporulation since several pathways between these two Firmicutes are conserved (109). The process of sporulation in *C. difficile* consists of four morphogenetic stages: (1) formation of a septum that separates the forespore (smaller compartment) from the mother cell (larger compartment) in a process of asymmetric cell division (Fig. 1); (2) engulfment of the forespore by the mother cell; (3) formation of the coat, cortex and inner membrane of the forespore; (4) cell lysis of the mother cell resulting in the release of the desiccated, stress-resistant chromosome-storing mature spore (109, 110).

The structure of *C. difficile* spores is similar to that of other endospore-forming pathogens like *B. subtilis*. The center of the spore is a pyridine-2, 6-dicarboxylic acid (DPA)-rich core that contains the genomic DNA, messenger RNA (mRNA), ribosomes and proteins. DPA, chelated with calcium (CaDPA) renders the spores heat-resistant (110). An inner membrane surrounds the core, followed by the peptidoglycan cortex and the protein-rich coat (110). Certain *C. difficile* strains (e.g. strain 630) produce a cysteine-rich exosporium morphogenetic protein (CdeC)-rich exosporium layer surrounding the coat (Fig. 1) (109-112). *cdeC*-/- mutant spores are more sensitive to ethanol and heat treatment suggesting that the exosporium further enhances resistance of spores to common disinfectants and heat (112). Furthermore, the spore coat and exosporium may have an

impact on adherence and germination (112-116). For example, spore coat protein *cotE*-/- mutants showed significant reduction in colonization and virulence in hamsters (114).

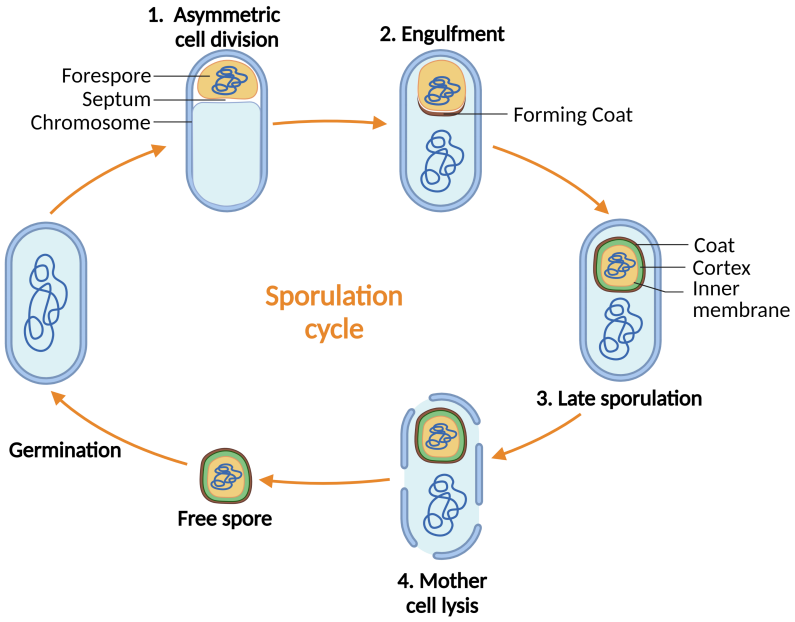


Figure 1. *Clostridioides difficile* Sporulation. The sporulation cycle has four morphogenetic phases: (1) the formation of a septum at on pole of the bacterium forming a forespore in a process called asymmetric cell division; (2) the forespore is engulfed by the mother cell; (3) the coat, cortex and inner membranes of the forespore are created; (4) the mother cell lyses and releases a mature spore (109, 110). *Created with BioRender*.

The exact environmental signals that trigger sporulation in *C. difficile* are unknown but may be related to nutrient deprivation or stressors identified via quorum sensing as in *B. subtilis* (109). Additional factors involved are CodY and catabolite control protein A (CcpA). These are transcriptional regulators that repress *C. difficile* sporulation factors (117, 118). CodY is a guanosine triphosphate (GTP)-sensing DNA-binding protein and CcpA is a LacI family DNA-binding protein that senses the availability of carbohydrates (117, 118). Both CodY and CcpA regulate stage 0 sporulation protein A (Spo0A), an essential factor for *C. difficile* sporulation (117-120). *C. difficile* strains that lack the *spo0A* gene fail to produce spores (121). Sensor histidine kinases (SHKs) phosphorylate and activate Spo0A which, in turn, regulates the entire network of genes implicated in the process of sporulation (122). In *C. difficile* strain 630, five SHKs have been identified: CD1352, CD1492, CD1579, CD1949 and CD2492 (122). The process by which Spo0A is phosphorylated is not well-described but evidence shows at least one SHK, CD1579, auto-phosphorylates and directly transfers a phosphate to Spo0A (122).

The formation of aero-tolerant dormant spores allows the dissemination and persistence of the pathogen, but disease is initiated by the metabolically-active vegetative form (123). Upon ingestion, a serine protease from the cold-shock protein (Csp) family, CspC, found on the surface of *C. difficile* spores binds to primary bile acids (PBAs) which include cholate, taurocholate, glycocholate and deoxycholic acid (DCA), and co-germinants which include amino acids (L-glycine or L-histidine) or divalent cations (Ca^{2+} or Mg^{2+}) (124-128). Kochan et al. demonstrated in *ex vivo* assays that removal of Ca^{2+} from mouse ileal contents resulted in abolishment of germination (128). Binding of a PBA to CspC results in the release of CaDPA from the spore core, thus re-hydrating the core and resuming metabolism (126). It is unclear whether CaDPA is released by direct activation of DPA channels or by the degradation of the cortex (109). It has been shown that Csps and spore cortex lytic enzymes (SCLEs), more specifically CspC, CspA, CspB and SleC are involved in the degradation of the spore cortex and are essential for *C. difficile* germination (126, 129-131). Binding of a germinant and co-germinant to CspC activates CspB resulting in the proteolytic cleavage of pro-SleC into active SleC which then hydrolyzes the spore cortex (109, 110, 132). Despite recent advances in our understanding of *C. difficile* sporulation and germination, much is still unclear. The triggers for *C. difficile* sporulation, the roles of co-germinants and CspA in *C. difficile* germination, the method by which CspA and CspB are transported into the spore and the mechanism by which CspC activates CspB are all current knowledge gaps (109, 110, 132).

In addition to the Csps and SleC, two additional molecules contribute to *C. difficile* germination: GerG (CD0311) protein and GerS lipoprotein (133, 134). The lack of *gerG* and *gerS* greatly impair germination as evidenced by the reduced responsiveness to germinants, decreased incorporation of Csps into spores, and failure to degrade the cortex (133, 134).

1.8.2 Toxins A, B and CDT

After germination, most strains of *C. difficile* start releasing TcdA (308 kDa) and TcdB (270 kDa) encoded in the pathogenicity locus (PaLoc) that includes genes for TcdR, TcdC and TcdE (1). *tcdR* and *tcdC* encode genes that regulate toxin expression (activator and repressor, respectively) (135, 136). Hypervirulence has been associated with the deletion of *tcdC* in certain strains such as the NAP1/B1/027 strain (47, 137). *tcdE* encodes a gene involved in facilitating toxin secretion (138). When nutrients are limited, *C. difficile* enters the stationary phase and starts

expressing TcdA and TcdB in the presence of short-chain fatty acids (SCFAs), e.g. butyrate (139, 140). Toxin synthesis is inhibited by glucose, amino acids (e.g., proline or cysteine), butanol and biotin (117, 141-143). TcdA and TcdB have four homologous domains: a biologically active N-terminal GTD, a cysteine protease domain (CPD), a delivery domain and a C-terminal RBD, and are overall 48% identical in their amino acid sequence (144, 145).

The RBDs of TcdA and TcdB contain repetitive sequences called combined repetitive peptides (CROPs) located between residues 1,833 – 2,710 and 1,834 – 2,366, respectively (144, 145). These toxins can be neutralized by antibodies raised against the RBD highlighting their role in pathogenicity in CDI (146-148). TcdA binds to GalNAc-(1,3)-β-Gal-(1,4)-β, and Lewis I, X, and Y glycan sequences on human intestinal epithelial cells (149, 150). Glycoprotein gp96 was also identified as a minor receptor for TcdA as evidenced by a partial block of TcdA toxicity by use of small interfering RNA (siRNA) directed to gp96 or anti-gp96 antibody (151). Receptors of TcdB include chondroitin sulfate proteoglycan 4 (CSPG4) and Wnt receptor Frizzled (FZD) identified by CRISPR/Cas9 genome wide screenings (152). CSPG4 plays a role in cell adhesion, proliferation and migration, and regulates the response to growth factors (153). Wnt signaling through FZD is important for stem cell and progenitor cell regulation, embryonic development, and cancer (154). The non-canonical Wnt signaling pathways require Rho and Rac proteins which are targets of TcdB (144, 154). However, it is unknown whether or not TcdB has an effect on these pathways (144). Knockout FZD7-/- mice have reduced disease severity after TcdB injection (155).

Cellular uptake of TcdA and TcdB occurs by endocytosis that is PACSIN2- and clathrin-dependent, respectively (156, 157). TcdA and TcdB then release the GTD into the cytosol of target cells (158). To release bioactive GTD, TcdA and TcdB undergo proteolytic cleavage behind a highly conserved leucine residue (Leu542 and Leu543, respectively) carried out by the CPD in the presence of inositol hexakisphosphate ($InsP_6$) (144, 158).

The GTDs of TcdA and TcdB (residues 1 – 542 and 1 – 543, respectively) target Rho/Ras proteins (RhoA/B/C, Rac1 and Cdc42) and inactivate them by quasi-irreversible glucosylation (at threonine 35 and 37) using uridine diphosphate (UDP)-glucose as a co-substrate (144). Additional targets of TcdA but not TcdB include Rap2 and R-Ras2 (144). These proteins are important for the regulation of migration, phagocytosis, intracellular trafficking, transcription, cell cycle progression and apoptosis (159). Glucosylation of Rho proteins prevents association of Rho

proteins with their respective effector proteins and blocks subsequent signal transmission resulting in pathogenic effects (160).

Certain strains of *C. difficile* (~20%), e.g. the NAP1/B1/027 strains, express an additional toxin: CDT (144, 161). CDT is a two-component actin ADP-ribosylating toxin (161). CDTa is the enzymatic component and CDTb is the binding component (161). The N-terminal of CDTa interacts with CDTb and the C-terminal contains the ADP-ribosyltransferase activity (161). CDTb has four domains: (I) the activation domain, (II) membrane insertion and pore formation domain, (III) oligomerization domain and (IV) receptor binding domain (161). CDT binds to lipolysis-stimulated lipoprotein receptor (LSR) expressed in liver, intestinal, lung and kidney cells via CDTb domain IV (162). Then, Domain I is proteolytically cleaved to activate oligomerization resulting in the formation of heptamers on the surface of target cells by domain III (144, 163). CDTa binds to CDTb and the CDT/LSR complex is endocytosed to a low pH compartment (144). Here, CDT inserts into the vesicle membrane (domain III of CDTb) and translocates to the cytoplasm with the help of intracellular folding proteins such as heat shock protein (HSP) 90, peptidyl-prolyl *cis-/trans*-isomerase cyclophilin A and FK-506-binding protein 51 (144).

In the cytosol, CDT ADP-ribosylates monomeric G-actin which binds to the polymerizing ends of actin filaments (4). ADP-ribosylated actin cannot polymerize with unmodified actin and therefore acts as a capping protein (4). The other end of actin filaments can still depolymerize (4). Eventually, all actin is depolymerized resulting in drastic changes in cell morphology and function (e.g. migration, endocytosis, and secretion) (4). The actin cytoskeleton also acts to limit the growth of microtubules without which cells produce long membrane protrusions through continuous microtubule formation (4). Moreover, the growth of microtubule depends on septins which are regulated by Cdc42, a target of TcdA and TcdB (144, 164). These microtubule protrusions have been shown to facilitate the transport of vesicles containing extracellular matrix (ECM) proteins (e.g. fibronectin) and enhance adherence of *C. difficile* to target cells, thus facilitating colonization (144). The importance of CDT in CDI pathogenicity is unknown but its expression is associated with higher mortality in patients (4, 165).

1.8.3 Non-toxin virulence factors

Several non-toxin virulence factors also play a role in *C. difficile* pathogenesis. These factors include surface layer or S-layer proteins (SLPs), cell wall proteins (CWPs), polysaccharides (PSI, PSII, and PSIII), and flagellar proteins (137).

SLPs have been detected in all *C. difficile* strains (166). *C. difficile* surface layer consists primarily of high molecular weight (HMW) and low molecular weight (LMW) protein subunits obtained by the cleavage of SlpA by Cwp84 and that play a role in adherence (166, 167). HMW and LMW have been shown to adhere to HEp-2 cells *in vitro*, and murine and human gastrointestinal tissues (167). More importantly, anti-SlpA antibodies or pre-treatment of cells with SlpA block adherence (167, 168). A well-characterized CWP is the CwpV (169). The C-terminal of CwpV is highly variable among *C. difficile* strains and there are at least 5 antigenically distinct types (169). However, its function, which is to promote bacterial aggregation, is conserved across all sub-types (169). Thus, CwpV is thought to play a role in biofilm formation (169). The biofilm shelters vegetative *C. difficile* cells from environmental stresses such as antibiotics, antibodies and oxygen, and provides a shielded environment for sporulation (137, 169).

Next, polysaccharides found on the cell surface are also important virulence factors (160). There are three in *C. difficile*: PSI, PSII and PSIII. All ribotypes have been identified to express polysaccharides with NAP1/B1/027 expressing PSII most abundantly (160). Furthermore, PSI-, PSII- and PSIII-specific immunoglobulin G (IgG) antibodies have been detected in the sera and PSII-specific IgA antibodies in the feces of CDI patients (170, 171). Several groups are currently exploring the potential use of PSII conjugates as vaccine candidates in various animal models (pig, mouse, and hamster) (172, 173).

The expression of flagella and the role it plays in *C. difficile* pathogenesis is controversial. Some studies have shown that flagellar expression (genes *fliC* and *fliD*) by certain strains of *C. difficile* is associated with decreased toxin expression and adherence. For example, the *fliC* mutant of a non-epidemic strain 630 (630Δ*erm*) increased transcription of 4 genes: *tcdA, tcdB, tcdE* and *tcdR*. Consequently, this mutant also produced more TcdA and TcdB (137, 160, 174). This can be explained by the presence of high levels of intracellular cyclic dimeric guanosine monophosphate (c-di-GMP) which repressed the expression of flagellar components by binding to riboswitch upstream of the *flgB* operon, and expression of *tcdA* and *tcdB (175)*. Baban et al. demonstrated that the *fliC* and *fliD* mutants of 630Δ*erm* adhered to Caco-2 cells more strongly than the wild-

type (WT) (176). In contrast, *fliC* and *fliD* mutants of NAP1/B1/027 strain R20291 adhered less than the WT (176). This inconsistency continues in *in vivo* studies as well. Some studies have demonstrated that *fliC* and *fliD* mutants of 630Δ*erm* are more virulent than the WT strain in hamsters while others have shown no difference between the mutants and the WT strain (174, 177). The flagella is also implicated in the formation of a biofilm (178). High levels of c-di-GMP promote the expression of type IV pili which, in turn, promotes biofilm formation (178, 179).

To summarize, the ability of *C. difficile* to sporulate and synthesize toxins (TcdA, TcdB and CDT) greatly contribute to *C. difficile* transmission, persistence and pathogenesis (1).

This project focused on the GTD and RBD of TcdB.

1.9 Immune response to *Clostridioides difficile*

1.9.1 Innate Immune Response

The innate immune response against acute CDI constitutes of the physical barrier, the microbiota and the immune cells (neutrophils, eosinophils, macrophages, mast cells, innate lymphoid cells (ILCs), and dendritic cells (DCs)) (180). The physical barrier not only consists of the cells that make the epithelial barrier but also the mucus layer (produced by goblet cells) that minimizes contact with luminal microorganisms (180). Furthermore, Paneth cells (i.e. highly specialized epithelial cells) and certain commensal bacteria produce antimicrobial peptides (AMPs) (defensins and cathelicidin LL-37) that further prevent infection by commensal microorganisms and some enteric pathogens, and enhance the effects of certain antibiotics against *C. difficile* (181). For example, reuterin, an antimicrobial compound produced by *Lactobacillus reuteri*, a member of the microbiota, promotes the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), enhances susceptibility of *C. difficile* to vancomycin and metronidazole, and inhibits toxin production by *C. difficile* (182). In addition to pro-inflammatory cytokines and chemokines, intestinal epithelial cells (IECs) also release ROS and RNS that limit dissemination of gut commensals, inhibit toxin self-cleavage and cell entry by S-nitrosylation (nitric oxide (NO)), and alter *C. difficile* metabolism which affects cell viability (182, 183). Interestingly, S-nitrosylation of toxins is enhanced in the presence of $InsP_6$ (183). Certain members of the gut microflora convert PBAs (necessary for *C. difficile* germination) to secondary bile acids, thus inhibiting germination of *C. difficile* spores (184). Most importantly, gut commensals help maintain intestinal homeostasis which has implications for CDI. IECs and tissue-resident immune

cells (e.g. macrophages and DCs) constantly sample the intestinal contents for pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and flagellin via pattern recognition receptors (PRRs) (e.g. toll-like receptors (TLRs)) (185). This continuous sampling helps maintain a population of inducible regulatory T cells (iTregs) and limit the accumulation of pro-inflammatory T helper 17 cells (Th17) (186). The balance in iTreg: Th17 ratio helps to maintain gastrointestinal homeostasis (186). Macrophages, DCs and IECs release interleukin (IL)-10 and transforming growth factor β (TGF-β) which inhibit inflammation and induce differentiation of iTregs (186, 187). The 'normal' gastrointestinal microbiota also induces the production of IgA from plasma cells for protection against certain pathogens, further inhibiting inflammation (188). With antibiotic treatment (the major risk for CDI) the microbiota can be remarkably altered (i.e. dysbiosis) which in turn causes an imbalance in the iTreg: Th17 ratio due to lack of PAMPs in the gastrointestinal environment (189). Th17 cell differentiation is induced whereas the number of iTregs is reduced, and a pro-inflammatory environment is created (189).

Inflammation is exacerbated in CDI (180). Upon ingestion, *C. difficile* spores germinate and release TcdA and TcdB (144, 180). These toxins then bind their respective cell surface receptors on human colonocytes and cause the dissociation of tight junctions (144). Increased vascular permeability and reorganization of the actin cytoskeleton structure leads to cell death, loss of epithelial barrier integrity and greater exposure to the gastrointestinal microflora (180). The NF-κB and activator protein 1 (AP-1) pathways are activated resulting in the release of pro-inflammatory mediators such as IL-1β, IL-2, IL-6, IL-8, IL-15, IL-16, IL-17A, macrophage inflammatory protein 2 (MIP-2) and tumor necrosis factor α (TNF-α) (180, 189). The levels of these mediators are significantly increased in CDI patients and they play a role in the recruitment, activation, differentiation of immune cells. More specifically, IL-1β and IL-6 promote Th17 differentiation (190, 191). IL-15 promotes T cell and natural killer (NK) cell activation and proliferation (190). IL-16 recruits and activates CD4+ cells (T cells, monocytes, eosinophils, and DCs) (190). IL-8 is a potent neutrophil chemoattractant (192). Studying the pattern of cytokines expressed in sera of CDI patients has helped to understand disease severity. For example, Abhyankar et al. showed that higher levels of IL-8, IL-6 and TNF-α are associated with increased risk for mortality (191). Additionally, a study by Yu et al. identified that higher levels of interferon (INF)-γ and IL-5 are associated with mild-to-moderate CDI whereas elevated IL-2 and IL-15 levels are associated with severe CDI (190).

As mentioned, cytokines and chemokines help recruit, activate and differentiate immune cells (180). Neutrophils are the first cells to arrive at the site of infection upon the release of IL-8 (192, 193) . A strong neutrophilic infiltration in the wall of the colon is a hallmark of CDI. The role of neutrophils in CDI is poorly understood but these cells have been known to phagocytose pathogens, and release immune mediators (i.e. cytokines and chemokines) and AMPs (194). Patients with severe CDI have larger numbers of neutrophils in the blood and are at higher risk of mortality (195). However, neutrophils are crucial for protection against CDI as evidenced by the increased risk of mortality and recurrence in leukemia patients with neutropenia and in allogenic hematopoietic stem cell transplant patients, respectively (196, 197). Neutrophil ablation experiments in animal models have yielded inconsistent results however. Use of anti-Ly6G^{+} antibodies in mice increased the risk of mortality but use of anti-CD18 in rabbits or anti-MIP-2 in rats resulted in reduced TcdA-induced cytotoxicity (198-200). Ly6G+ (in mice) and CD18 are cell surface markers of neutrophils and MIP-2 is a neutrophil chemoattractant in rats (198-200). Hence, the full role of neutrophils in CDI is not yet completely understood but the presence of these cells is likely crucial for a protective immune response against *C. difficile*.

The little evidence acquired on the role of eosinophils in CDI suggests that early infiltration of these cells is protective against CDI (201, 202). Microbiota-derived IL-25 promotes eosinophil infiltration at the site of infection that can help to repair and maintain epithelial barrier integrity (202). Depletion of eosinophils with anti-SiglecF antibodies in mice results in increased risk of mortality in this model (202). Additionally, the CDT toxin produced by certain hypervirulent strains (e.g. NAP1/B1/027) has been shown to increase virulence of *C. difficile* by inhibiting the eosinophilic response (201). Binding of CDT to TLR2 on eosinophils suppresses their protective activity and mice that receive TLR2-/- eosinophils are better protected than WT mice (201). More recently, a study showed that eosinopenia (i.e. low count of eosinophils) and infection with a CDT-producing *C. difficile* strain increases the risk of mortality in CDI patients (203). Therefore, eosinophils are likely to play an important role in protection against CDI.

Current studies demonstrate that macrophages may not be protective in CDI because of their ability to exacerbate inflammation and retain spores but further studies are required to further understand their role (204, 205). Macrophages phagocytose *C. difficile* vegetative cells and spores, and release pro-inflammatory cytokines, including IL-1β and IL-6 in a MyD88- and TLR2-

dependent fashion (204). As mentioned previously, a robust inflammatory response against *C. difficile* is detrimental for the patient (180). Macrophage migration inhibition factor (MIF) is expressed by several cell types and tissues, including macrophages and monocytes, and plays a crucial role in driving inflammation (206). Jose et al. observed higher levels of circulating MIF in CDI patients compared to patients with diarrhea who were negative for *C. difficile* (207). Jose et al. also demonstrated that CDI in mice increases the level of MIF in the plasma and tissues (207). Using a neutralizing anti-MIF antibody decreased the inflammatory response, symptom severity and mortality in mice (207). More recently, it was discovered that CDI patients express high levels of macrophage inflammatory protein 1 α (MIP-1α) upon exposure to TcdA (208). MIP-1α is expressed primarily by macrophages in pro-inflammatory sites and is a chemokine that recruits macrophages, lymphocytes and eosinophils (209). Blocking TcdA-induced MIP-1α using a neutralizing antibody minimized tissue damage, reduced IL-1β mRNA expression, and prevented recurrent CDI (rCDI) and death in mice (208). Additionally, an *in vitro* study demonstrated that spores remain intact in the macrophages upon phagocytosis creating a reservoir for *C. difficile*, and are even cytotoxic to macrophages (205). Therefore, macrophages likely play a pathogenic role in CDI.

Mast cells are granulocytes that contain several inflammatory mediators, including histamine, nitric oxide (NO), and cytokines (210). In response to CDI, mast cells degranulate, release histamine and promote recruitment of pro-inflammatory cells (e.g. neutrophils) (189, 211, 212). The exact trigger for mast cell degranulation in the context of CDI is unknown (212). For effective immune cell infiltration, histamine increases epithelial permeability resulting in fluid accumulation in the intestinal lumen and diarrhea (212). Mast cells also release TNF-α and IL-8, pro-inflammatory cytokines that contribute to the infiltration of circulating immune cells (213, 214). Mast cell-deficient mice have significantly less fluid secretion and neutrophil infiltration, and thus, less inflammation in response to TcdA (212). Therefore, mast cells likely play a pathogenic role in CDI.

ILCs are another type of immune cell involved in the response to CDI (180). ILCs mimic effector functions of T cells but are activated by PAMPs, stress signals and mediators at the site of infection rather than specific antigens like T cells (215). Therefore, ILCs are highly reactive and one among the first effector cells to respond to external stressors (215). These cells are divided in

three categories: T-bet-expressing ILC1s, Gata-3/ROR-α-expressing ILC2s and RORγt/Ahr-expressing ILC3s which reflect the cytokine profiles of Th1, Th2 and Th17 cells, respectively (215). In the context of CDI, each of these categories of ILCs may play a role (180).

ILC1 cells respond to IL-12, IL-15 and IL-18 by producing IFN-γ and TNF-α, two cytokines involved in promoting inflammation by activating macrophages and inducing ROS production (215, 216). IFN-γ enhances TcdA-induced enteritis in mice (217) and, as mentioned previously, elevated levels of TNF-α in the serum of CDI patients are associated with a higher risk of mortality (191). Selective loss of ILC1s in *Rag1-/- Tbx21-/-* mice (i.e. mice that lack B and T cells, and T-bet, a transcription factor for the differentiation of ILC1 cells) increased disease severity and mortality (218). This increase was similar to that of Ragγc-/- mice (i.e. mice that lack B and T cells, and ILCs) (218). Therefore, despite their role in promoting inflammatory responses, ILC1 cells appear to be the main contributors in protection against acute CDI.

ILC2 cells respond to IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) released by the damaged epithelium, and produce IL-4, IL-5, and IL-13 that promote mucus production and aid in tissue repair (215, 216). ILC2 cells play a protective role in CDI as evidenced by the increase in survival of Ragγc-/- mice after transfer of ST2+ ILC2 cells (i.e. IL-33-sensitive ILC2 cells) compared to Ragγc-/- mice (30% vs 0%, respectively) (219). Furthermore, restoration of ILC2 cells in a Ragγc-/- mice favored accumulation of eosinophils which is protective against CDI and reduced neutrophil counts (219). This is equally relevant in humans as anti-IL-33 immunohistochemistry staining of colonic biopsies from CDI patients compared to those from healthy individuals demonstrated increased expression of IL-33 (219). Therefore, ILC2 cells also likely contribute to protection against CDI.

ILC3 cells express granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-17 and, predominantly, IL-22 after activation by IL-1β and IL-23. IL-22 maintains intestinal homeostasis by inducing production of AMPs and GM-CSF is crucial for the survival, differentiation and function of intestinal macrophages and DCs (216, 220). *Il22-/-* mice are more susceptible to CDI compared to WT mice as evidenced by increased disease severity and mortality rates (218). This study also showed that *Rag1-/-il17a-/-* mice do not have higher mortality rates post-challenge despite the lack of IL-17A (in addition to B and T cells) compared to WT mice (218). Together, this study suggests a minor role for ILC3 cells in protection against CDI compared

to ILC1 cells. However, this statement is debateable since several studies have shown conflicting results. Nakagawa et al. showed that knocking out (KO) IL-17A and IL-17F in mice increased resistance to CDI with a NAP1/B1/027 strain as evidenced by decreased mortality rates compared to WT mice (221). The levels of inflammatory mediators (IL-1β, GM-CSF, CXCL2 and IL-6) and neutrophil infiltration were also significantly decreased (221). Therefore, further studies are required to elucidate the role of ILC3 cells and, more specifically, the cytokines released by these cells (IL-22 and IL-17) in the host defense against CDI.

Dendritic cells (DCs) are known to be one of the major bridges between innate and adaptive immunity (222). These are APCs that uptake, process and present antigens to naïve T cells via major histocompatibility complex class II (MHC-II) molecules (222). *In vitro*, TcdB-intoxicated IECs release alarmins that activate DCs (223). Activated DCs phagocytose intoxicated IECs and release cytokines, including TNF-α which promotes apoptosis of intoxicated IECs and further aids in DC maturation (i.e. positive feedback loop) (223). TcdB-intoxicated IECs also promote infiltration of circulating DCs *in vivo* further enhancing inflammation (223). DCs also release IL-1β, indicating that these cells may also play a role in the formation of an inflammasome (224). In a recent study, Sun et al. used transgenic mice in which DCs could be conditionally depleted by administering diphtheria toxin (225). When DCs were depleted, these transgenic mice were unable to clear *C. difficile* from the intestine and bacterial dissemination into other organs was observed, potentially due to a compromised epithelial barrier (225). Macrophage infiltration was also reduced and overall, CDI severity was increased (225). Therefore, elucidating the precise role that DCs play in CDI requires further studies.

1.9.2 Adaptive Immune Response

A protective adaptive immune response, both antibody and cell-mediated, is important for protection against CDI recurrence (180).

After activation, B cells differentiate into memory B cells (MBCs) and plasma cells (226). The former play a role in a recall infection or recurrence (226). The latter produce three main Ig isotypes involved in the host defense against CDI: IgM, IgG and IgA (180, 226). Approximately 50-70% of healthy individuals have detectable serum IgG and IgA antibodies against *C. difficile* toxins (189, 227-229). IgM is the first to be produced in a humoral immune response and has lower affinity for the *C. difficile* antigens than other isotypes (230). Early appearance (day 3 post-onset

of diarrhea) of TcdA-specific serum IgM antibodies is associated with a decreased risk of recurrence in CDI patients (231). This study supports the importance of IgM in the host defense against *C. difficile*.

IgG is the most abundant immunoglobulin found in the body, representing 75% of total serum antibodies, and plays a role in the systemic immune response to CDI by enhancing opsonisation and activating the complement system (230, 232). Serum IgG titers against both toxins are higher in CDI patients with mild disease compared to severe CDI (233). Furthermore, several studies have shown a strong correlation between asymptomatic CDI and the lack of recurrences with high toxin-specific serum IgG titers compared to patients with relapsing CDI (231, 234-236). Additional evidence is provided by the work that led to the approval of bezlotoxumab, a TcdB-specific IgG1 monoclonal antibody, by the FDA for preventing rCDI (95, 237, 238). Hence, these studies validate the importance of IgG antibodies in the immune response against *C. difficile*.

IgA is a multimeric antibody that is secreted into the gut lumen and, thus, plays a role in mucosal immunity. IgA is, therefore, expected to play a role against *C. difficile* which is an enteric pathogen (188). Increased survival is seen in challenged hamsters after treatment with vancomycin and orally administered secretory IgA (pooled from human plasma) compared to vancomycin alone (239). In CDI patients, reduced fecal IgA titers and colonic IgA-producing cells is correlated with recurrence (234, 240). Several studies have shown that higher IgA titers correlated with milder CDI symptoms or asymptomatic carriage suggesting that mucosal immunity against *C. difficile* is important in the protection against severe CDI and disease recurrence (233, 241, 242).

Although innate and humoral responses undoubtedly play a role in recovery from CDI, adequate and well-balance T cell responses are likely to play a pivotal role in the prevention of recurrences. The lack of both B cells and T cells in mice (*Rag1-/-*) does not affect the resolution of the acute phase of CDI but is still associated with high mortality rates suggesting that the cellular adaptive immune response is important in full recovery and prevention of re-infection (218). B cells, as mentioned above, can differentiate into Ig-producing plasma cells or memory B cells that are activated in a recall infection (226). IgD-negative TcdA-specific MBCs were detected in CDI patients and the presence of antibodies in the serum and fecal samples suggests that plasma cells were also likely present (243).

Some of the T cell types involved in the host defense against CDI are T follicular helper cells (Tfh), T helper 1 (Th1) cells, Th17 cells, and iTregs. Naïve CD4+ T cells in the T cell zones of secondary lymphoid organs that encounter *C. difficile*-specific antigens, become activated (cytokines IL-6 and IL-21) and migrate to the B cell zone to activate follicular B cells are called Tfh cells (244). Tfh cells aid in the creation of germinal centers (GCs) where activated B cells go through rounds of isotype switching, somatic hypermutation, and rapid cellular division and in determining B cell fate (MBCs or plasma cells) (244). The presence of *C. difficile*-specific antibodies in CDI patients means that Tfh were involved since the lack of Tfh cells results in a defect in GC formation and antibody production (228, 229, 231, 233-236, 241-245). Naïve CD4+ T cells also differentiate into Th1, Th17 or iTreg cells in response to their cognate antigen, appropriate co-stimulation and cytokines (IFN-γ and IL-12; TGF-β, IL-6, IL-21 and IL-23; TGF-β and IL-2, respectively) (246, 247). These CD4 T cell subsets have different cytokine profiles and therefore, have different functions. The ability of a CD4 T cell to change subsets during the course of an infection (i.e. plasticity) complicates our ability to precisely describe the T cell response to CDI (248-250). Using flow cytometry to investigate the different T cell subsets found in the blood of CDI patients, Yacyshyn et al. observed a larger percentage of CD3+ CD4+ cells co-expressing Foxp3 and IL-17; IFN-γ and IL-17, IL-17 alone and Foxp3 alone which represent Th17/Treg, Th1/Th17, Th17, and Treg cells respectively, in patients with rCDI compared to patients who experience a single episode or controls (251). Therefore, CD4+ T cells are definitely involved in the adaptive immune response against CDI but whether they are protective or deleterious is still unknown.

To summarize, the immune response to *C. difficile* is complex. Key players that may or may not be protective against this pathogen include the gastrointestinal microbiota, cytokines and chemokines, innate immune cells (neutrophils, eosinophils, macrophages, mast cells, ILCs, DCs), immunoglobulins (IgM, IgG, IgA), B and T cells (MBCs, plasma cells, and Tfh, Th1, Th17, iTreg cells).

1.10 *Clostridioides difficile* infection vaccine development

Currently, no vaccine exists against *C. difficile*. Vaccines that target antibody induction against one or both of the toxins (TcdA and TcdB) are obviously attractive since CDI is a toxin-mediated disease. Three pharmaceutical companies (Pfizer, Sanofi Pasteur and Valneva Austria)

have developed adjuvanted, intramuscular vaccines using this approach that have undergone clinical trials. Alternative approaches include vaccine that prevent colonization (targeting SLPs or polysaccharides) and that induce mucosal responses.

1.10.1 Clinical trials

Three vaccines against *C. difficile* have entered clinical trials. Pfizer developed a genetically detoxified recombinant TcdA and TcdB toxoid vaccine (PF-06425090) given intramuscularly in three doses to adults aged 50 and over (252-254). This vaccine candidate completed its phase III clinical trial in December of 2021 (253). The results showed that the vaccine was safe and well-tolerated in humans. Although it reduced CDI severity overall and was 100% efficacious in preventing medically attended CDI, its efficacy to prevent primary CDI was only 30% and, therefore, it did not meet its primary endpoint (253). The company is now evaluating the next steps for this vaccine (253).

Sanofi Pasteur's vaccine consisted of formalin-inactivated TcdA and TcdB toxoids (Cdiffense) and was also given intramuscularly in three doses (255, 256). Sanofi Pasteur terminated its phase III clinical trial in December of 2017 as the interim analysis showed no protective efficacy and further clinical development of Cdiffense was stopped (255, 257).

Valneva developed a recombinant fusion protein consisting of the RBDs of both toxins (VLA84) given intramuscularly in three doses to adults aged 50 and over (258). VLA84 has completed phase II clinical trials in which the vaccine was shown to be immunogenic (i.e. generated IgG responses and neutralizing titers), and identified the dose and formulation with the highest seroconversion rates against both TcdA and TcdB which was the primary endpoint (259). Further development, however, has been on hold for >5 years was halted for unknown reasons (259).

1.10.2 Alternative approaches

Novel methods for vaccine development are emerging that focus on preventing *C. difficile* colonisation and inducing a mucosal response. Recently, Bradshaw's and Karyal's groups have both developed a vaccine against CD0873, a *C. difficile* colonisation factor (260, 261). CD0873 is a surface-exposed lipoprotein involved in adhesion of *C. difficile* to target cells (260, 261). Bradshaw et al. showed that mice immunized with recombinant CD0873 prevented long-term

colonization of *C. difficile* which correlated with increased secretory IgA (sIgA) responses (260). Karyal et al. immunized hamsters orally with CD0873 and these animals produced higher titers of sIgA and IgG; survived longer post-challenge with a NAP1/B1/027 strain (R20291*ermB*) of *C. difficile* and demonstrated reduced pathology in histological analyses of the cecum compared to naïve hamsters (261). The antibodies that were generated greatly inhibited adherence of *C. difficile* to Caco-2 cells (261).

A different approach to preventing colonization of toxigenic *C. difficile* was taken by Wang et al. who sought to generate a vaccine targeting both the toxins (GTD and CPD of TcdB, and RBD of TcdA) and adhesion factors using a non-toxigenic strain of *C. difficile* (NTCD) (CCUG37785) as a vector (NTCD_mTcd138) (262). This way, colonization by a toxigenic strain is inhibited by spatial and nutritional competition (263). Oral immunization with spores of NTCD_mTcd138 offered 100% protection to mice against challenge with a NAP1/B1/027 strain (UK6) (262). This vaccine was further modified to include the RBD of TcdB (strain NTCD_mTcd169) (264). Oral immunization of mice with NTCD_mTcd169 spores induced mucosal and systemic antibody responses; and protected 100% of mice from challenge with a NAP1/B1/027 strain (R20291) (264).

This project focused on generating a mucosal vaccine comprised of the GTD and RBD of TcdB using and attenuated bacterial vector (*Salmonella enterica* serovar Typhimurium strain YS1646) against NAP1/B1/027 and potentially all strains of *C. difficile*.

1.11 Summary of *Clostridioides difficile*

C. difficile is a leading cause of nosocomial diarrhea in the Western world representing 10-20% of cases (9). The CDC has classified this bacterium as an urgent public threat (9). CDI is transmitted primarily by the fecal-oral route and, usually, initiated after the germination of ingested spores when toxins are released (TcdA, TcdB and CDT) (1, 106). Current therapies are only partially effective as reflected by the high recurrence rate (15%-35%) highlighting the need for preventative measures such as a vaccine (92). Three vaccine candidates have undergone clinical trials but none has been approved for use against CDI in any jurisdiction (253, 255, 259). All three targeted TcdA and TcdB since CDI is a toxin-mediated disease and were given parenterally in multiple doses with adjuvants (253, 255, 259). Furthermore, the current understanding of the elicited host immune response remains incomplete. However, it is understood that the adaptive

immune response, particularly the humoral response, plays a central role in preventing CDI and rCDI (180, 226). More specifically, a mucosal response, particularly through the production of IgA antibodies, has been shown to provide considerable protection against CDI and rCDI (233, 234, 240, 241). Therefore, a vaccine eliciting a strong mucosal response would likely be effective against *C. difficile*.

1.12 *Salmonella enterica* serovar Typhimurium strain YS1646

Salmonella enterica (S. enterica) are rod-shaped, food-borne, Gram-negative gastrointestinal bacteria that cause salmonellosis with symptoms ranging from mild gastroenteritis to systemic disease (265, 266). The CDC estimate 1.35 million cases of salmonellosis annually in the U.S. alone predominantly affecting young children, the elderly and the immunocompromised with 26,500 hospitalizations and 420 deaths (267). Outbreaks are usually caused by *S. enterica* serovars Typhimurium (S. Tm) and Enteritidis (268). Salmonellosis is the 2nd leading cause of food-borne illnesses after norovirus (267). Despite its ability to cause disease, several research groups have succeeded in attenuating S. Tm to use as a vaccine vector (269).

Several studies using S. Tm as a vector for delivery of heterologous antigens (i.e. immunogens of other pathogens) have been reported in animals. For example, oral immunization of mice with S. Tm expressing the *Bacillus anthracis (B. anthracis)* protective antigen (PA) provides full protection against lethal challenge with *B. anthracis* spores (270). Oral immunization of mice with S. Tm expressing a recombinant enterotoxigenic *Escherichia coli (E. coli)* K99 fimbriae demonstrated promising results (267, 271, 272). These research groups exploited S. Tm's type 3 secretion system (T3SS) to secrete the heterologous antigen. S. Tm's T3SS are encoded on *Salmonella* pathogenicity islands (SPI)-I and SPI-II (268).

S. Tm as a vaccine vector has many theoretical advantages. It is easy to manipulate and manufacture, and has high carrying capacity (105). The flagellin of S. Tm (sFliC) acts as an adjuvant by activating the TLR5 signaling pathway (273). Wang, S et al. reported that the inclusion of sFliC in their chimeric toxin vaccine (along with RBDs of TcdA and TcdB, GTD and CPD of TcdB) protected mice from lethal challenge with a hypervirulent strain of *C. difficile* (273). TLR5 activation by *Salmonella*-derived flagellin in mice results in delayed *C. difficile* growth and toxin production (273, 274). S. Tm also produces LPS which activates TLR4 further enhancing the immune response (105). Importantly, as CDI is limited to the gut, S. Tm infection causes local

inflammation and influx of neutrophils in the gut (275). S. Tm targets cells of the gut mucosa and has the potential to elicit strong cellular and humoral immune responses in the colonic mucosa (267, 276, 277).

S. Tm strain YS1646 has chromosomally deleted *purI* and *msbB* genes, components of the purine biosynthesis pathway and the LPS synthesis pathway, respectively (105, 269, 278). YS1646 was initially developed in the early 1990s as a treatment for metastatic melanoma and other solid tumors. Clinical trials demonstrated that intravenous administration of up to $3.0x10^8$ colony forming units (CFU)/m^2 of YS1646 was tolerated in humans. Unfortunately, the clinical trials were halted for futility (i.e. no benefits) (269). Recent studies in the Ward/Ndao labs have demonstrated the efficacy of YS1646 for the delivery of several different heterologous antigens. Hassan et al. reported almost complete protection against *Schistosoma mansoni* after vaccination with YS1646 expressing cathepsin B (278). Additionally, Chen, G et al. used YS1646 to generate a vaccine against *Schistosoma japonicum* which showed 75% protection in mice (279).

In parallel, Winter et al. from the War/Ndao labs developed a vaccine containing the RBDs of TcdA and TcdB (rbdA and rbdB, respectively) (105). Briefly, the rbdA was cloned into the pQE_30 plasmid under the control of a constitutive promoter pagC and secretory signal SspH1 (pagC_SspH1_rbdA). The same was done for rbdB but using a SPI-II-specific promoter and secretory signal (sspH2_SspH2_rbdB). WT YS1646 strain was transformed by electroporation with the plasmids to generate strains expressing rbdA, rbdB and no antigen (i.e. empty plasmid). Recombinant antigens (rrbdA and rrbdB) were generated in *E. coli*. Expression of the antigens was confirmed by Western blotting. C57Bl/6J female mice (six-to-eight weeks) were vaccinated using different routes and schedules. The multimodal schedule (recombinant antigen on day 0 intramuscularly (i.m.), and YS1646 antigen-expressing strain orally (p.o.) on days 0, 2 and 4) was confirmed to give the best serologic response in a short time. Five weeks post-vaccination, C57Bl/6J mice were challenged with $2x10^5$ to $2x10^7$ CFU/mouse of freshly grown *C. difficile* (historical strain VPI 10463) and they observed 100% survival in all mice except for the phosphate-buffered saline (PBS) control and the p.o. only groups. Serum (3-4 weeks post-vaccination) and intestine (5 weeks post-infection) analyzed by enzyme-linked immunosorbent assay (ELISA) demonstrated both antigen-specific systemic IgG and intestinal IgA responses compared to the PBS control (105).

1.13 Hypothesis and aims

Similarly to these candidate RBD vaccines, the YS1646 strain was also transformed with a plasmid containing GTD (pagC_SspH1_GTD) and recombinant GTD (rGTD) was produced in *E. coli* by a previous student in the Ward/Ndao lab. To test immunogenicity of these GTD-targeting reagents, mice were vaccinated using the multimodal schedule described above and preliminary results showed the induction of a detectable anti-TcdB IgG response (data not published). The protective efficacy of the vaccine was not tested. We hypothesized that delivery of GTD and RBD of TcdB using an S. Tm strain YS1646 vector would protect against lethal challenge with NAP1/B1/027 strain of *C. difficile*. We aimed to modify a murine model for NAP1/B1/027 challenge and to evaluate the protective efficacy against NAP1/B1/027 challenge of a GTD-expressing YS1646 vaccine compared to a more traditional vaccine targeting the RBD of the more common TcdB *C. difficile* toxin.

CHAPTER 2: METHODS, RESULTS and DISCUSSION

2.1 METHODS

2.1.1 Bacterial strains and culture conditions

S. enterica serovar Typhimurium strain YS1646 (Δ*msbB2* Δ*purI* Δ*Suwwan xyl*-; ATCC 202165; ATCC, Manassas, VA) was obtained from Cedarlane Labs (Burlington, ON). Transformed YS1646 bacteria (electroporation; 2 µL of plasmid at 3.0 kV, 200 Ω, and 25 µF; GenePulser XCell, BioRad, Hercules, CA, USA) were cultured and plated on Luria broth (LB) containing 50 ug/mL ampicillin (Wisent, St.-Bruno, QC, Canada). *Escherichia coli* BLR (de3) (Novagen, Millipore Sigma, Burlington, MA) was used for production of rGTD and rrbdB. pET28b plasmids encoding GTD DNA (pET28b-GTD) sequence or rbdB (pET28b-rbdB) DNA sequence were introduced in *E. coli* strains by heat shock (2 µg of plasmid with 50 µL of cells in 50˚C water bath for 30 seconds). Transformed *E. coli* were grown on petri dishes with Luria broth (LB) containing 50 µg/mL kanamycin (Wisent). *C. difficile* strain VPI 10463 and clinical NAP1/B1/027 strains were obtained from Cedarlane Labs and from Dr. Vivian Loo at the Montreal General Hospital (Montreal, QC), respectively. *C. difficile* were cultured in pre-reduced meat broth (Sigma-Aldrich, St.-Louis, MO) containing 0.1% (wt/vol) L-cysteine (Sigma-Aldrich) in an anaerobic jar at 37˚C for 24h or 72h for VPI 10463 and NAP1/B1/027 strains, respectively. For

colony counts, serially-diluted *C. difficile* cultures were plated onto pre-reduced brain heart infusion (BHIS) (BD Biosciences, Mississauga, ON, Canada) plates containing 0.1% (wt/vol) L-cysteine (Sigma-Aldrich) and incubated in an anaerobic jar at 37˚C for 24h.

2.1.2 Recombinant protein expression, purification and quantification

Competent *E.coli* BLR(de3) (Sigma-Aldrich) cells were transformed with the pET28b plasmid containing the GTD or rbdB gene sequences. A mixture of 2 µL of pET28b-GTD or -rbdB and 50µL of freshly thawed commercial BLR(de3) were incubated on ice for 30 minutes, heat shocked in a 50˚C water bath for 30 seconds then left on ice for 5 minutes. Cells were cultured at 37˚C in a shaking incubator for 1h at 200 rpm after addition of 200 µL of LB broth (Wisent). The culture (100 µL) was plated on LB-kanamycin (Wisent) selection plates and grown at 37˚C for 24h. Transformed cells were stored in aliquots in LB-glycerol (15%) at -80˚C.

For purification of recombinant protein, transformed cells were cultured in 30 mL of LB-kanamycin (30 µg/mL) (Wisent) at 37˚C for 24h in a shaking incubator at 200-250 rpm. LB-kanamycin (600 mL; 30 µg/mL) was inoculated with 24mL of the overnight culture and incubated in a shaking incubator at 37˚C until OD_{600} of between 0.5-0.6 was achieved. The culture was induced with 0.5mM Isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen, Carlsbad, CA) at 37˚C for 24h in a shaking incubator at 200-250 rpm. The culture was centrifuged at 4˚C for 30 minutes at 3000xg. The supernatant was discarded and the cell pellets were re-suspended in 12mL denaturing solution (100mM NaH_2PO_4, 10mM Tris-HCl, 8M Urea, pH = 8.0) and lysed by freeze-thaw (-80˚C or liquid nitrogen). The cells were centrifuged at 4˚C for 15-30 minutes at 20 000xg and the supernatant was collected in a conical tube. The proteins were purified using Ni-nitrilotriacetic acid (NTA) affinity chromatography (Qiagen, Limburg, Netherlands) via the histidine (His)-tag using a series of 3M denaturing washes (urea) with decreasing pH. Proteins were visualized using GelCode Blue Stain Reagent (Thermo Fisher Scientific, Eugene, OR) or Coomassie Blue staining in SDS-PAGE gels. Eluates were dialyzed in 4L of PBS for 1h twice and then overnight. A Western blot using monoclonal antibody anti-GTD (Emergent Biosolutions, Gaithersburg, MD) or anti-His (Sigma-Aldrich) was performed to confirm the presence of rGTD or rrbdB, respectively.

Purified rGTD and rrbdB were quantified using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit following the manufacturer's instructions (Thermo Fisher Scientific). Briefly,

10 µL of bovine serum albumin (BSA; Sigma-Aldrich) standard (0 µg/mL – 2000µg/mL) or the purified protein (0.1 and 0.01 fold dilutions, and neat) were added to each well of a flat-bottom 96-well plate in triplicate. Working reagent (200 µL) was added to each well. The plate was incubated at 37˚C for 30 minutes. Optical density at 562 nm (OD_{562}) was measured using the Tecan Infinite F200 Fluorescence Microplate Reader (McGill University Health Center Research Institute (RI-MUHC), Montreal, QC).

2.1.3 Mice

All animal procedures were approved by the McGill University's Animal Care Committee and were performed in accordance to the guidelines of the Canadian Council on Animal Care. Female 6- to 8-week-old C57BL/6 mice, obtained from Jackson Laboratories (Bar Harbor, ME, USA), were housed (3-5/cage) at the Animal Resource Division (ARD) in the RI-MUHC in a pathogen-free environment and according to routine rodent husbandry procedures. Euthanasia was performed by first anesthetizing mice with isoflurane in 100% O_2 until unresponsive, and then asphyxiation with 100% CO_2 followed by cardiac puncture or cervical dislocation.

2.1.3.1 Vaccination

For the booster study to evaluate the immunogenicity of rGTD, mice received three i.m. doses of 10 µg of rGTD adjuvanted with 250 µg of aluminum hydroxide gel (alum; Alhydrogel; Brenntag BioSector A/S, Frederikssund, Denmark) in 50 µL of PBS on days 0, 21 and 35. A 26-gauge needle was used to administer the i.m. dose in the gastrocnemius muscle (Fig. 2).

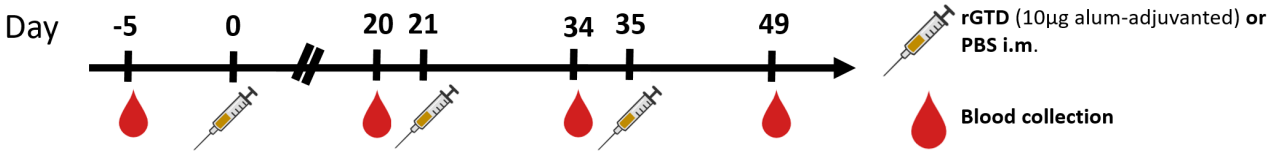
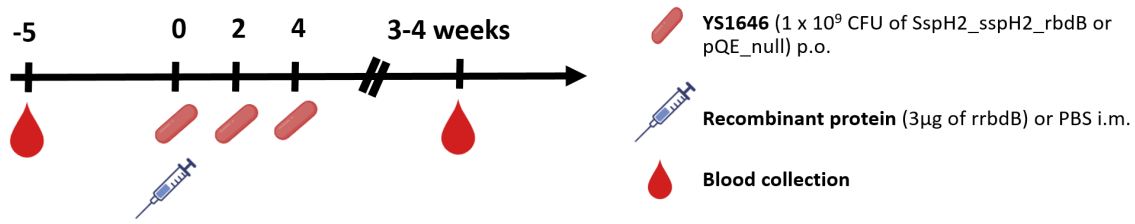


Figure 2. rGTD booster study timeline. A single i.m. dose of 10 µg rGTD was administered on days 0, 21 and 35. Blood was collected by saphenous vein bleed on days -5, 20, and 34, and by cardiac puncture on day 49. *Created with BioRender.*

To evaluate the efficacy of the rbdB vaccine against NAP1/B1/027 challenge, we used 4 groups of mice: PBS (control), rbdB i.m., rbdB p.o. and rbdB multimodal group (Fig. 3B). The rbdB i.m. group received a single dose i.m. of rrbdB adjuvanted with 250 µg of alum (Brenntag BioSector A/S) in 50 µL of PBS on day 0 (Fig. 3A). A 26-gauge needle was used to administer

the i.m. dose in the gastrocnemius muscle. The rbdB p.o. group received three p.o. vaccinations delivered by gavage containing a total of $1x10^9$ CFU of plasmid-strain YS1646 strain expressing rbdB (SspH2_sspH2_rbdB) in 200 µL of PBS on days 0, 2 and 4 (Fig. 3A). Strain SspH2_sspH2_rbdB has been evaluated for its immunogenicity and protective efficacy against VPI 10463 (105). The rbdB multimodal group received a single i.m. dose of rrbdB on day 0 and three p.o. doses of SspH2_sspH2_rbdB on days 0, 2 and 4 (Fig. 3A).

A



B

Vaccination groups				
Group	Number of mice	i.m. vaccination (3 µg alum-adjuvanted)	p.o. vaccination (1 x 10^9 CFU/mouse of YS1646)	Shorthand
1	8	PBS	PBS	PBS
2	10	rrbdB	pQE_null	rbdB i.m.
3	10	PBS	SspH2_sspH2_rbdB	rbdB p.o.
4	10	rrbdB	SspH2_sspH2_rbdB	rbdB multimodal

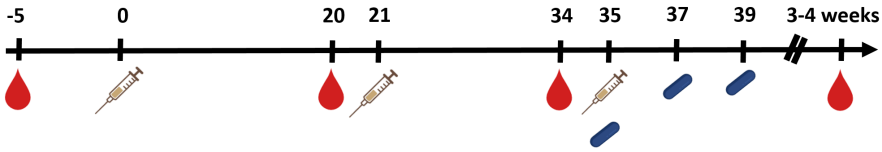
Figure 3. rbdB vaccination A) schedule and B) groups. Group 1 received PBS i.m. (D0) and p.o (D0, D2, D4). Group 2 received one dose of 3µg of rrbdB adjuvanted with 250µg of alum (D0), and three p.o doses of YS1646 expressing no antigen (pQE_null) (D0, D2, D4) (rbdB i.m.). Group 3 received PBS i.m. (D0) and three p.o. doses of pagC_SspH1_rbdB (D0, D2, D4) (rbdB p.o.). Group 4 received one dose of rrbdB i.m. (D0) and three p.o. doses of pagC_SspH1_rbdB (D0, D2, D4) (rbdB multimodal). Blood was collected by saphenous vein bleed D-5, and 3 to 4 weeks post-vaccination. Mice were kept alive for NAP1/B1/027 challenge. *Created with BioRender*.

To evaluate the efficacy of the 'solo' GTD and combined GTD + rbdB vaccines against NAP1/B1/027 challenge, we used 7 groups of mice: PBS (control), GTD i.m., GTD p.o., GTD multimodal, GTD + rbdB i.m., GTD + rbdB p.o., and GTD + rbdB multimodal (Fig. 4C). The GTD i.m. group received three i.m. doses of 6 µg of rGTD adjuvanted with 250 µg of alum (Brenntag BioSector A/S) in 50 µL of PBS on days 0, 21 and 35 (Fig. 4A). A 26-gauge needle was used to administer the i.m. dose in the gastrocnemius muscle. The GTD p.o. group received three

doses of PBS on days 0, 21 and 35; and three p.o. doses of $1x10^9$ CFU of a plasmid-based YS1646 strain expressing GTD (pagC_SspH1_GTD) in 200 µL of PBS on days 35, 37 and 39 (Fig. 4A). The 'solo' GTD multimodal group received three i.m. doses of rGTD on days 0, 21 and 35; and three p.o. doses of pagC_SspH1_GTD on days 35, 37 and 39 (Fig. 4A). The GTD + rbdB i.m. group received three i.m. doses of rGTD on days 0, 21 and 35; a single dose of rrbdB on day 35 and three p.o. doses of $1x10^9$ CFU of pQE_Null on days 35, 37 and 39. The GTD + rbdB p.o. group received three i.m. doses of PBS on days 0, 21 and 35; and three p.o. doses of each pagC_SspH1_GTD and SspH2_sspH2_rbdB (total of $1x10^9$ CFU) on days 35, 37 and 39. The combined GTD + rbdB multimodal group received three i.m. doses of rGTD on days 0, 21 and 35; one i.m. dose of rrbdB on day 35; three p.o. doses of each pagC_SspH1_GTD and SspH2_sspH2_rbdB (total of $1x10^9$ CFU) on days 35, 37 and 39 (Fig. 4B).

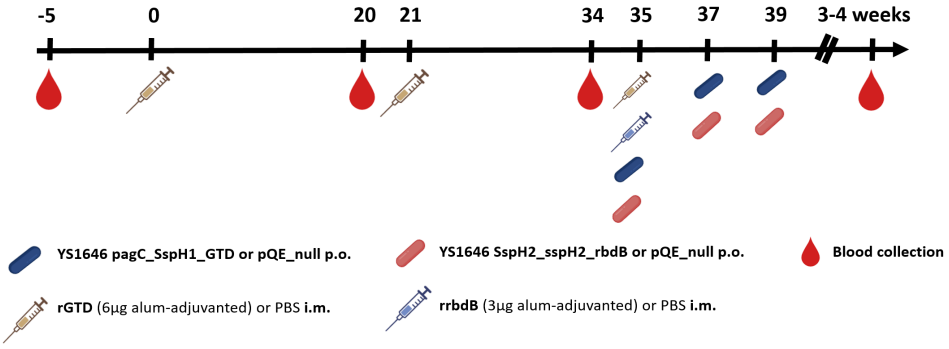
A

GTD vaccine



B

GTD + rbdB vaccine



C

Vaccination groups				
Group	Number of mice	i.m. vaccination	p.o. vaccination (total of 1 x 10^9 CFU/mouse of YS1646)	Shorthand
1	8	PBS	PBS	PBS
2	10	rGTD	pQE_null	GTD i.m.
3	10	PBS	pagC_SspH1_GTD	GTD p.o.
4	10	rGTD	pagC_SspH1_GTD	GTD multimodal
5	10	rGTD + rrbdB	pQE_null	GTD + rbdB i.m.
6	10	PBS	pagC_SspH1_GTD + SspH2_sspH2_rbdB	GTD + rbdB p.o.
7	10	rGTD + rrbdB	pagC_SspH1_GTD + SspH2_sspH2_rbdB	GTD + rbdB multimodal

Figure 4. The GTD and GTD + rbdB vaccination A-B) schedule and C) groups. A) GTD vaccine. Group 1 received PBS i.m. (D0, D21, D35) and p.o. (D35, D37, D39). Group 2 received 6µg of rGTD alum-adjuvanted in 50µL of PBS (D0, D21, D35) and YS1646 strain expressing no antigen (pQE_null; D35, D37, D39) (GTD i.m.). Group 3 received PBS i.m. and YS1646 strain pagC_SspH1_GTD (D35, D37, D39) (GTD p.o.). Group 4 received rGTD i.m. (D0, D21, D35) and pagC_SspH1_GTD (D35, D37, D39) (GTD multimodal). **B) GTD + rbdB vaccine.** Group 5 received rGTD i.m. (D0, D21, D35) + 3 µg of rrbdB alum-adjuvanted i.m. (D35), and pQE_null (D35, D37, D39) (GTD + rbdB i.m.). Group 6 received PBS i.m. (D0, D21, D35) and pagC_SspH1_GTD + SspH2_sspH2_rbdB (D35, D37, D39) (GTD + rbdB p.o.). Group 7 received rGTD (D0, D21, D35) + rrbdB (D35), and pagC_SspH1_GTD + SspH2_sspH2_rbdB (D35, D37, D39) (GTD + rbdB multimodal). Blood was collected D-5, D20, D34 and 3 to 4 weeks post-vaccination. Mice were kept alive for NAP1/B1/027 challenge. *Created with BioRender*.

2.1.3.2 Serum collection

Baseline and post-vaccination serum samples were collected by saphenous vein bleed or submandibular vein bleed in Microtainer serum separator tubes (Sarstedt, Nübrecht, Germany). At the end of the study, mice were euthanized and serum samples were collected by cardiac puncture. Serum was separated by centrifugation at 8000xg for 10 minutes at room temperature, aliquoted and stored at -20˚C until used in assays.

2.1.3.3 *Clostridioides difficile* challenge

One week prior to antibiotic treatment, acetic acid at a concentration of 2.15 µL/mL (vol/vol) was added to the drinking water of mice. An antibiotic cocktail consisting of metronidazole (0.215 mg/mL; Sigma-Aldrich), gentamicin (0.035 mg/mL; Wisent), vancomycin (0.045 mg/mL; Sigma-Aldrich), kanamycin (0.400 mg/mL; Wisent) and colistin (0.042 mg/mL; Sigma-Aldrich) was added to the drinking water at 8 days prior to infection NAP1/B1/027 for 3 consecutive days after which mice were put back on regular drinking water. Mice received an i.p. dose of clindamycin (Sigma-Aldrich) at a concentration of 32 mg/kg of body weight in 200 µL of PBS using a 26-gauge needle 72h prior to infection NAP1/B1/027. The dose of the *C. difficile* administered was estimated on the day of the infection (OD_{600}) as freshly cultured *C. difficile* was used. The challenge dose was administered in 200 µL of pre-reduced meat broth. The exact dose was calculated using a standard of CFU/mL vs. OD_{600} 24h post-infection. Mice were monitored and scored 1 to 3 times daily for approximately 2 weeks on their weight loss, posture, activity, coat, diarrhea and eyes/nose (280). The humane endpoint was established as a score of ≥14 or weight loss >20% of their initial weight at which point mice were euthanized (Fig. 5).

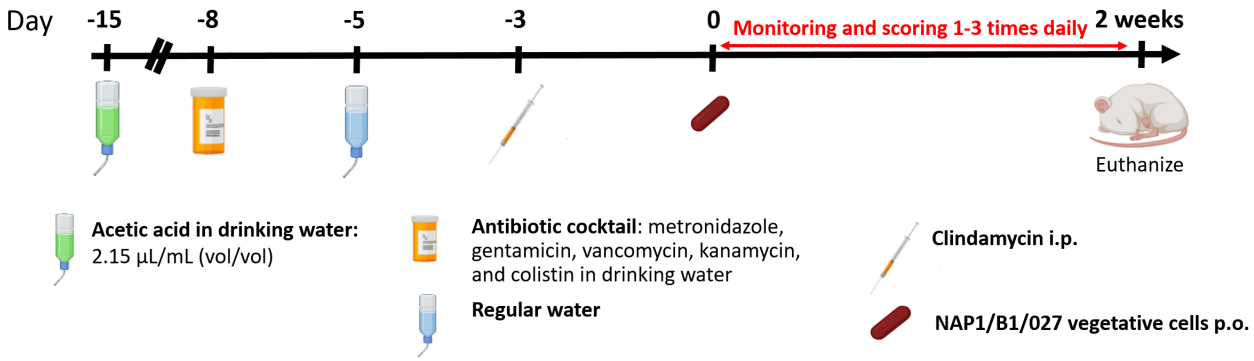


Figure 5. Murine model of *Clostridioides difficile* strain NAP1/B1/027 challenge. Mice received acetic acid in drinking water on day -15, an antibiotic cocktail (metronidazole, gentamicin, vancomycin, kanamycin, and colistin) in drinking water from day -8 to -5, a single i.p. dose of clindamycin 72h prior to p.o. infection with freshly-grown NAP1/B1/027 vegetative cells. Mice were monitored and scored 1 to 3 times daily for 2 weeks after which they were euthanized. *Created with Biorender*.

2.1.4 ELISA

Whole toxin B (List Biologicals, Campbell, CA) was used to coat the wells of a U-bottom high-binding 96-well ELISA plates (Greiner Bio-One, Frickenhausen, Germany). A standard curve was generated using mouse IgG (Sigma-Aldrich). The wells were coated with 50 µL of toxin B (2 µg/mL), or IgG standards overnight at 4˚C in 100mM bicarbonate/carbonate buffer (pH 9.5). After washing the wells 3 times with PBS, the plates were blocked with 150 µL/well of blocking buffer (2% BSA; Sigma-Aldrich) in PBS-Tween 20 (0.05%; Fisher Scientific)) for at least 1h at 37˚C. Serum samples were added after being heat-inactivated (56˚C water bath for 30 minutes) and diluted (1:50 in blocking buffer). Intestinal samples were added neat. All samples, including the standards, were added in duplicate at 50 µL/well. The plates were incubated for 1h at 37˚C, washed 4 times with PBS then incubated for 30 minutes at 37˚C after adding 75 µL/well of horse radish peroxidase (HRP)-conjugated anti-mouse IgG (1:20 000 in blocking buffer; Sigma-Aldrich). After washing the plates 6 times with PBS, 100 µL/well of 3, 3', 5, 5'-tetramethylbenzidine (TMB) detection substrate (Millipore, Billerica, MA) was added. The reaction was stopped after 15 minutes with 50 µL/well of 0.5M H_2SO_4. The plates were read at 450 nm using an EL800 microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT). The concentrations were estimated by extrapolation from the standard curve.

2.1.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.

2.2 RESULTS

2.2.1 Optimization of the protocol for NAP1/B1/027 culture

The lab protocol requires the media, meat broth (Sigma-Aldrich, St.-Louis, MO) 10% w/v L-cysteine (Sigma-Aldrich), to be reduced (i.e. oxygen-deprived) for at least 48h prior to inoculation in an anaerobic jar (Thermo Fisher Scientific, Eugene, OR) containing an anaerobic pack (Thermo Fisher Scientific) and an oxygen indicator (Thermo Fisher Scientific) (Table 1) (105, 281-283). The inoculum is cultured at 37˚C at 200 RPM for 24h in a shaking incubator and the absorbance is measured using a spectrophotometer at a wavelength of 600 nm. An OD is obtained as an approximate measure of the turbidity, i.e. cell density (284). Unfortunately, early work with this protocol demonstrated that it was not optimal for the NAP1/B1/027 strain. Culture of a historical strain, VPI 10463, using this protocol routinely resulted in an OD of >2.00 compared to only 0.630 for NAP1/B1/027 (Table 1, Experiment #001). Experiments # 003-013 where we changed the medium to brain heart infusion-supplemented (BHIS) (281-283), and increased the reduction time (72h-120h), the concentration of L-cysteine (15%-50%), and the number of inoculations were failures. However, our hypothesis that NAP1/B1/027 needed more time to grow proved to be correct. When the growth time was increased from 24h post-inoculation to 72h (Table 1, Exp. #014), we observed an increase in the OD value from 0.630 to 1.059 and this was consistent when repeated (Table 1, Exp. #014-017). The optimized protocol required inoculation with 100 µL of the glycerol stock in pre-reduced (48h) meat broth (10% w/v L-cysteine) and culture for 72h in a shaking incubator at 37˚C, 200 rpm.

Table 1. Protocol optimization for NAP1/B1/027 culture.

Initial protocol (optimal for VPI 10463 culture)								
	Reducing time	% L-cysteine	Media	Number of inoculations	Growth time	Optical density (OD)		
	48h	10%	Meat broth	1	24h	>2.00		
NAP1/B1/027 culture optimization								
Experiment #	**Reducing time**	**% L-cysteine**	**Media**	**Number of inoculations**	**Growth time**	**OD**	**Next steps**	**Changes to protocol**
1	48h	10%	Meat broth	1	24h	0.63	Increase growth time: 48h	
2	48h	10%	Meat broth	1	**48h**	1.453	Different media: BHIS	Grow in 48h
3	48h	10%	**BHIS**	1	48h	1.213		Keep meat broth
4	48h	10%	Meat broth	1	48h	–	Extra inoculation 24h later	
5	48h	10%	Meat broth	**2**	48h	1.007		Perform 2 incolutions, 24h apart
6	48h	10%	Meat broth	2	48h	–	Increase reduction time: 72h, 96h, 120h	
7	**72h, 96h, 120h**	10%	Meat broth	2	48h	–	Increase L-cysteine concentration	Keep reducing time to 48h
8	48h	**0%, 10%, 15%, 20%, 50%**	Meat broth	2	48h	–	Added >1 beads in 1st (4 beads) and 2nd (2 beads) inoculation	Keep L-cysteine concentration to 10%
9	48h	10%	Meat broth	2	48h	–	Inoculating from a glycerol stock	
10	48h	10%	Meat broth	2	48h	–	Adding 100ul of glycerol stock	
11	48h	10%	Meat broth	2	48h	0.86		
12	48h	10%	Meat broth	2	48h	0.862	Repeat	
13	48h	10%	Meat broth	2	48h	0.771	Repeat	
14	48h	10%	Meat broth	1	**72h**	1.059	Increased growth time	
15	48h	10%	Meat broth	1	72h	1.045	Repeat	
16	48h	10%	Meat broth	1	72h	1.055	Repeat	
17	48h	10%	Meat broth	1	72h	1.052	Repeat	
Optimized protocol for NAP1/B1/027 culture								
	Reducing time	**% L-cysteine**	**Media**	**Number of inoculations**	**Growth time**	**OD**		
	48h	10%	Meat broth	1	72h	>1.0		

Note: changes in the protocol are shown in red.

2.2.2 Standard curve of NAP1/B1/027 culture

We cultured NAP1/B1/027 using the optimized protocol. We performed 2-fold serial dilutions of the culture (1/32 of neat to neat culture) and measured the OD of each dilution (Figure 1A) (104, 284). We then performed 10-fold dilutions of each 2-fold dilution and plated dilutions ranging from 10^{-4} to 10^{0} (Fig. 6A). Using the formula shown in Figure 1A, we calculated the CFU/mL (284, 285). We showed the standard curve with the linear equation $Y = 5.14 \times 10^{5}X - 7.17 \times 10^{4}$ and the R squared (R^2) value of 0.908 suggesting a strong correlation (Fig. 6B). This standard curve was used to estimate the dose administered to mice on the day of the infection in CFU/mL.

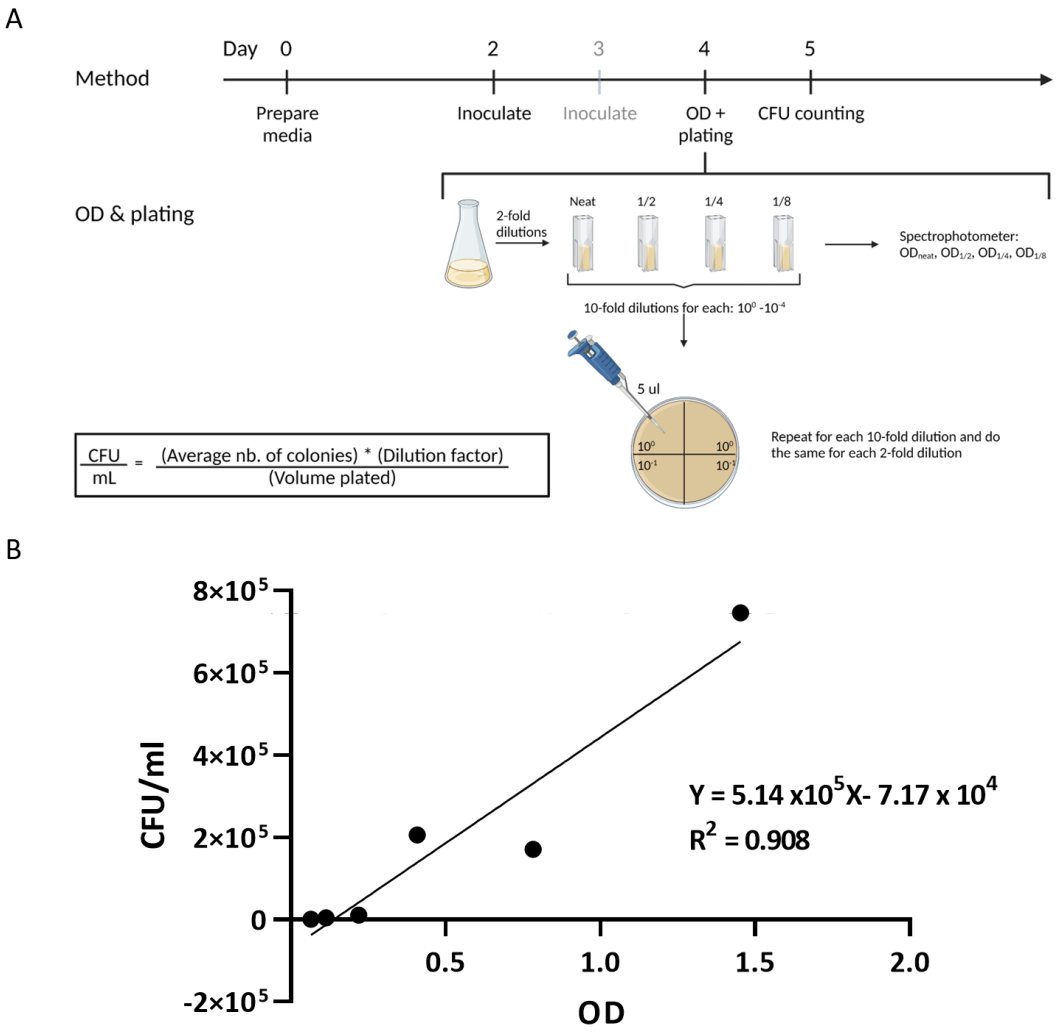


Figure 6. Standard curve of NAP1/B1/027 colony forming units (CFU)/mL relative to the optical density of the culture. A) The method used for serially-diluting the culture and plating NAP1/B1/027. B) The standard curve of CFU/mL relative to the OD of the culture. *Created with BioRender.*

2.2.3 Optimization of NAP1/B1/027 challenge dose in mice

Female 6-8 weeks-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) housed (3-5/cage) in the ARD in pathogen-free conditions were infected by gavage with four different doses of NAP1/B1/027: 0 CFU/mouse, 80 CFU/mouse, 1.2 x 10^2 CFU/mouse and 3.06 x 10^3 CFU/mouse. All mice that received 0 CFU of NAP1/B1/027 typically survived with minimal to no symptoms (Figure 7B) for the duration of each experiment (representative experiment shown in Fig. 7A). Overall, ~60% of mice that received 80 CFU succumbed to infection (Fig. 7A) with 40% becoming moribund within 48 hours (Fig. 7B). The percent mortality of mice challenged with 1.2 x 10^2 CFU/mouse and 3.06 x 10^3 CFU/mouse was typically ≥80% (Fig. 7A) with almost all of the animals experiencing severe symptoms (clinical score of ≥11) by the second day after oral

dosing (Fig. 7B). Based on these results, we determined the optimal CFU count for lethal challenge with NAP1/B1/027 to be between 1.2 x 10^2 CFU/mouse and 3.06 x 10^3 CFU/mouse.

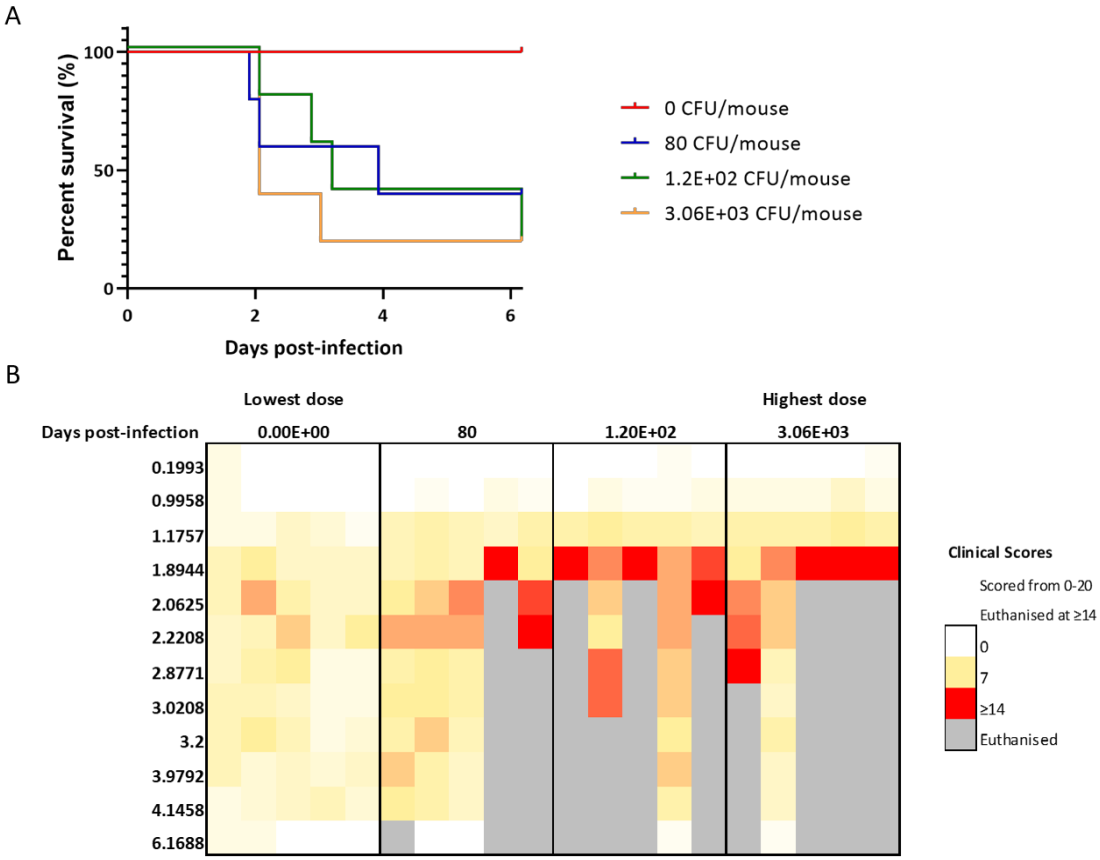


Figure 7. Mice succumbed to NAP1/B1/027 infection in a dose-dependent manner. A) Survival and B) clinical scores are shown (n = 5) for four groups of mice challenged orally with different CFU doses of NAP1/B1/027 strain: 0 CFU/mouse (red), 80 CFU/mouse (blue), 1.2 x 10^2 CFU/mouse (green) and 3.06 x 10^3 CFU/mouse (yellow). Each column represents one mouse as it progresses through the infection. Mice were clinically scored one to three times daily for up to 7 days post-infection. Mice with a score of ≥14 or a >20% loss of body weight were considered at a humane endpoint and were euthanized.

2.2.4 Immunogenicity of recombinant GTD

Female 6-8 weeks-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) housed (3-5/cage) in the ARD in pathogen-free conditions received 10µg of rGTD in 50 µL of PBS on days 0, 21 and 35 in the gastrocnemius muscle. Serum was collected to measure TcdB-specific IgG titers by ELISA on days -5, 20, 34 and 49. We demonstrated that three i.m. doses of rGTD at 10µg significantly increased TcdB-specific IgG titers to up to an average of 1.36 x 10^4 ng/mL which was approximately 21-fold higher than PBS control group (average of 6.40 x 10^2 ng/mL) (Fig. 8). Mice that received one dose i.m. of rGTD did not elicit an immune response (Fig.

8). Two doses i.m. of rGTD increased 2.7-fold TcdB-specific IgG titers (average of 1.73 x 10^3 ng/mL) compared to the PBS control but this increase was not significant (Fig. 8). These results suggest that the rGTD is immunogenic.

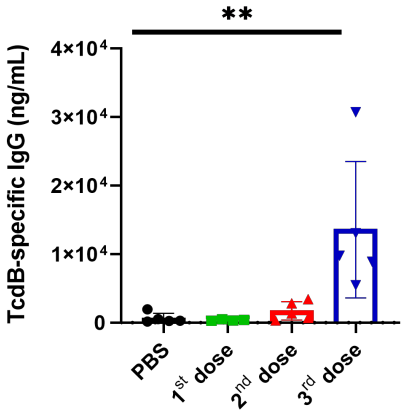


Figure 8. Three doses of rGTD elicited a significant increase in TcdB-specific IgG titers. Mice received 10 µg of rGTD intramuscularly in 50 µL of PBS on days 0, 21 and 35. An ELISA was performed using serum collected on days -5, 20, 34 and 49. The Mann-Whitney test was used to compare all groups to the PBS control group. $^{**}P<0.01$.

2.2.5 Efficacy of the rbdB vaccine against NAP1/B1/027 challenge

Female 6-8 weeks-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) housed (3-5/cage) in the ARD in pathogen-free conditions were vaccinated with PBS (control group), rbdB i.m. (D0), rbdB p.o. (D0, D2, D4) or both (i.e. multimodally). Winter et al. demonstrated that the multimodal schedule was the most efficacious method of vaccination against VPI 10463 (105). All groups were challenged orally with freshly grown NAP1/B1/027 at a dose of 6.4 x 10^2 CFU/mouse which is within the range anticipated to result in ~80% mortality (Fig. 7). Mice were monitored 1 to 3 times daily for weight loss, clinical score and death. In this experiment, 50% of mice that received rbdB p.o. (D0, D2, D4) or PBS succumbed to infection by ~72 h post-infection (Fig. 9A). The surviving mice experienced severe symptoms but recovered by ~48 h post-challenge (Fig. 9B). All mice (100%) that received rbdB i.m. (D0) survived the challenge and none experienced severe illness. Mice that were vaccinated multimodally with rbdB (i.m./p.o.) had an 80% survival rate (Fig. 9A). The proportion of mice vaccinated multimodally that were severely ill was 20% (Fig. 9B). This proportion is lower than mice from the PBS control and rbdB p.o. (D0, D2, D4) groups (40%) (Fig. 9B). These results suggest that mice that receive at least an i.m. dose of rbdB (rbdB i.m. and rbdB multimodal groups) are protected against NAP1/B1/027 challenge with an efficacy of at least 80% (Fig. 9A).

rbdB i.m. (D0) and multimodally elicited TcdB-specific IgG titers at concentrations of 5.78 x 10^4 ng/mL and 5.34 x 10^4 ng/mL, respectively, 3 post-vaccination (i.e. pre-challenge) which was significantly higher than the PBS control and rbdB p.o. (2.49 x 10^3 ng/mL and 1.78 x 10^3 ng/mL, respectively) (Fig. 9C). Only the rbdB p.o. (D0, D2, D4) group had no detectable serum IgG response (Fig. 9C). There was no significant difference in TcdB-specific IgG titers between rbdB i.m. (D0) and multimodal groups (Fig. 9C). These results suggest a strong correlation between high serum TcdB-specific IgG titers pre-challenge and protection against NAP1/B1/027 challenge.

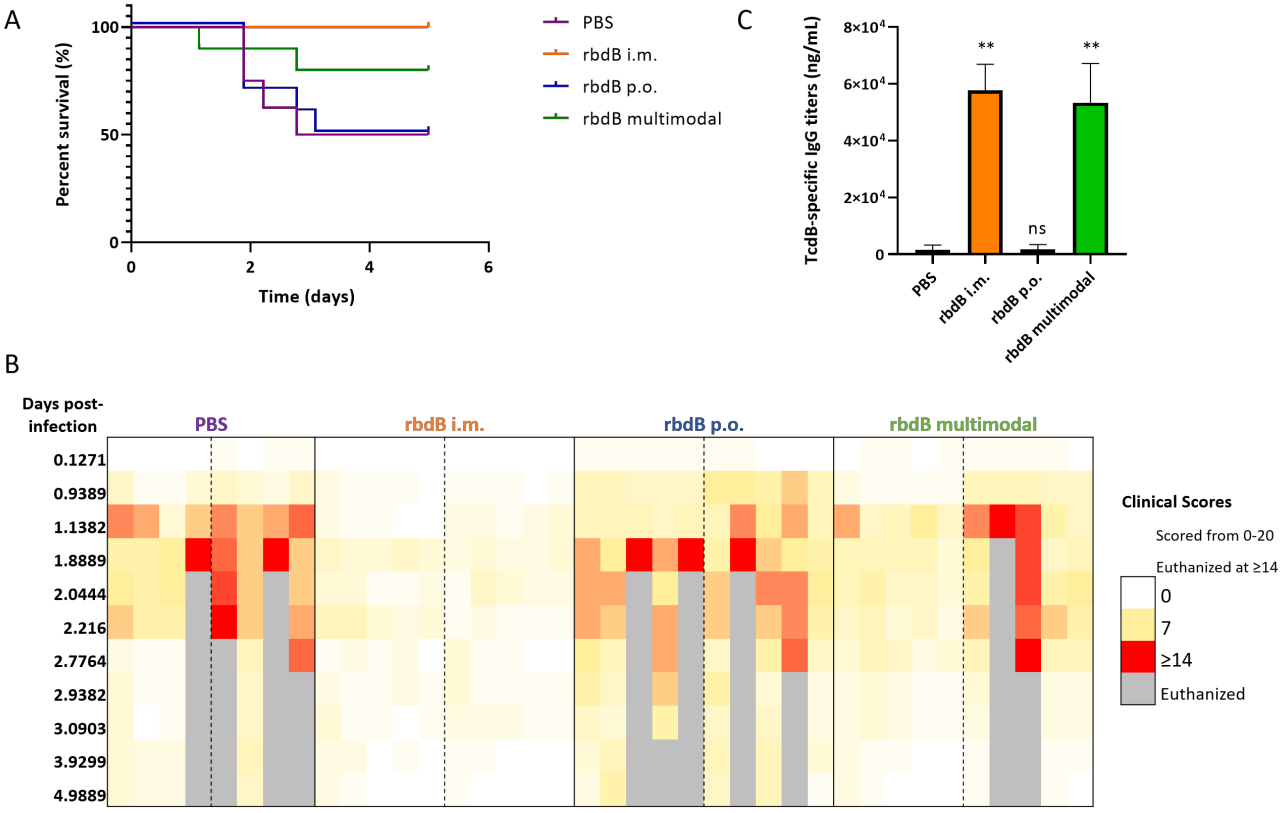


Figure 9. Immunization with receptor binding domain of toxin B (rbdB) protects mice from lethal challenge with NAP1/B1/027. A) Survival curve; B) clinical scores and C) TcdB-specific IgG titers (ng/mL) in serum are shown (n = 8 to 10) for four vaccination groups: PBS (purple), rbdB i.m. (orange), rbdB p.o. (blue) and rbdB multimodally (i.e. rbdB i.m./p.o.) (green) challenged with 6.4 x 10^2 CFU/mouse of NAP1/B1/027. Mice received 3 µg of rrbdB i.m. on day 0; and a total of 1x10^9 CFU of PagC_SspH1_GTD and SspH2_SspH2_rbdB orally (p.o.) on days 0, 2 and 4. Mice were bled on days -5 and 21 to measure serum IgG titers using an ELISA. Mice were clinically scored one to three times daily for up to 5 days post-infection. Mice with a score of ≥14 or a ≥20% loss of body weight were considered at a humane endpoint and were euthanized. The dotted lines indicate mice in the same group but in different cages. The Mann-Whitney test was used to compare all groups to the PBS control group. ns = non-significant; $^{**}P < 0.01$.

2.2.6 Efficacy of the GTD and GTD + rbdB vaccines against NAP1/B1/027 challenge

Female 6-8 weeks-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) housed (3-5/cage) in the ARD in pathogen-free conditions were vaccinated with PBS (control group), GTD i.m. (D0, D21, D35), GTD p.o. (D35, D37, D39) or GTD multimodally (i.m. D0, D21, D35/p.o. D35, D37, D39). The combination groups consisted of GTD (D0, D21, D35) + rbdB (D35) i.m.; GTD + rbdB p.o. (D35, D37, D39) or GTD + rbdB multimodally (i.m./p.o.). We challenged all groups with NAP/B1/027 at a dose of 2.41 x 10^3 CFU/mouse 4 weeks post-vaccination which is within the range anticipated to result in ~80% mortality (Fig. 7A). In this experiment, 50% of mice who received PBS succumbed to infection by ~48h post-infection. The mortality rates in the 'solo' GTD p.o. and combined GTD + rbdB p.o. were 20% and 10%, respectively (Fig. 10A). The proportion of severely ill mice in the 'solo' GTD p.o. and combined GTD + rbdB p.o. groups was lower than the control group (10% vs. 37.5%) (Fig. 10B). All mice (100%) that received GTD i.m.; GTD multimodally; GTD + rbdB i.m.; and GTD + rbdB multimodally survived the infection (Fig. 10A) with the maximum clinical score given to be 6, indicating a very mild disease (Fig. 10B). These results suggest that the combined GTD + rbdB vaccine given i.m. or multimodally can improve survival of mice post-infection compared to the 'solo' rbdB multimodal vaccine. However, the 'solo' GTD vaccine given i.m. or multimodally may be sufficient in protecting mice against lethal challenge with NAP1/B1/027 (Fig. 10A).

TcdB-specific IgG titers were significantly increased 3 weeks after vaccination with GTD i.m. (1.17 x 10^4 ng/mL); GTD multimodally (7.3 x 10^3 ng/mL); GTD + rbdB i.m. (2.13 x 10^4 ng/mL); GTD + rbdB p.o. (7.3 x 10^2 ng/mL); and GTD + rbdB multimodally (3.15 x 10^4 ng/mL) compared to the PBS control group (Fig. 10C). The GTD p.o. group had no detectable serum IgG responses (Fig. 10C). Interestingly, the combined GTD + rbdB multimodal vaccine elicited significantly higher TcdB-specific IgG titers than the 'solo' GTD multimodal vaccine ($P < 0.01$) (Fig. 10C) but both vaccines had 100% efficacy against lethal challenge with NAP1/B1/027 (Fig. 10A). The combined GTD + rbdB i.m. vaccine elicited slightly higher IgG titers than the 'solo' GTD i.m. vaccine but this difference failed to reach statistical significance (Fig. 10C). TcdB-specific IgG titers from the combined GTD + rbdB p.o. group were 7 to 30 times lower than all other vaccination groups, except for the 'solo' GTD p.o. group. These results suggest that an increase in TcdB-specific IgG titers is correlated with high efficacy.

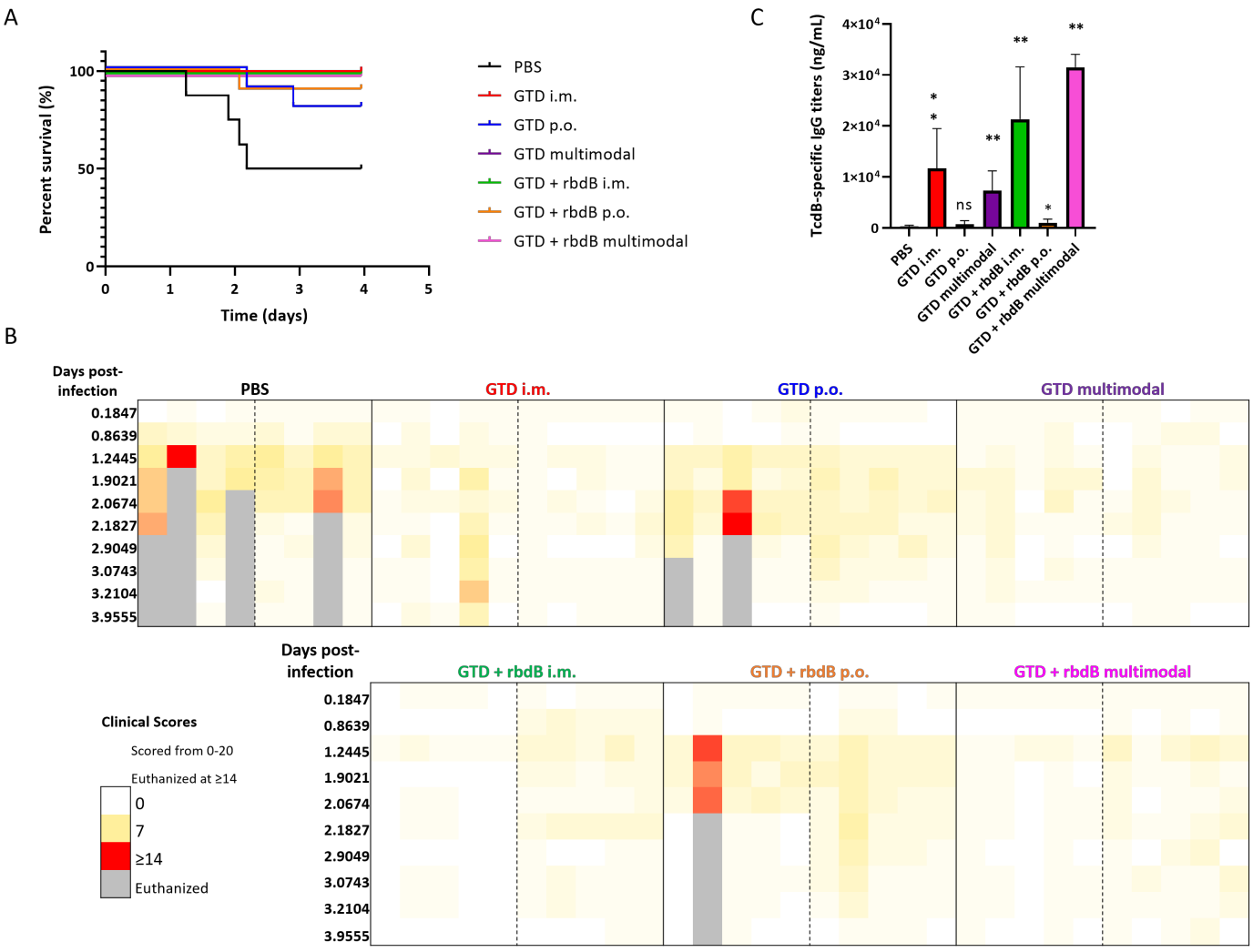


Figure 10. Immunization with the glucosyltransferase domain (GTD) of toxin B protects mice from lethal challenge with NAP1/B1/027. A) Survival curve; B) clinical scores and C) TcdB-specific IgG titers (ng/mL) in serum are shown (n = 8 to 10, no repeats) for 7 vaccination groups: PBS (black); GTD i.m. (red); GTD p.o. (blue); GTD multimodal (purple); GTD + rbdB i.m. (green); GTD + rbdB p.o. (orange); and GTD + rbdB multimodal (pink) challenged with 2.41 x 10^3 CFU/mouse of NAP1/B1/027. Mice received 6 µg of GTD i.m. in 50 µL of PBS (D-35, D-14, D0); 3 µg of rbdB (D0); and 1x10^9 CFU of GTD p.o. or rbdB p.o. (D0, D2, D4). The GTD + rbdB p.o. group received 5 x 10^8 CFU of each antigen. Mice were bled on days -40 and 3 weeks post-vaccination to measure serum IgG titers using an ELISA. Mice were clinically scored 1-3 times daily for up to 4 days post-infection. Mice with a score of ≥14 or a ≥20% loss of body weight were considered at a humane endpoint and were euthanized. The dotted lines indicate mice from the same group but in different cages. The Mann-Whitney test was used to compare all groups to the PBS control group. ns = non-significant; *$P < 0.05$; **$P < 0.01$.

2.3 DISCUSSION

C. difficile was first observed and characterized by Hall and O'Toole in 1935 and ~40 years later, this bacterium was considered an etiological agent for PMC (5, 99). Today, *C. difficile* is a leading cause of nosocomial diarrhea in developed countries (10%-20% of cases) and is classified as an urgent public threat by the CDC (9). The emergence of hypervirulent epidemic strains (most notably, NAP1/B1/027 strains) and the high recurrence rate (15-35%) despite treatments (e.g. antibiotics, FMT, bezlotoxumab), highlights the need for development of preventative strategies vaccines (47, 92). After years of effort and many clinical trials including two major Phase 3 studies, there is still no vaccine for CDI. These efforts were not in vain however (253, 255, 259). We learned that targeting *C. difficile* toxins is a promising approach but that the intramuscular route of administration may not be effective for this particular gastrointestinal pathogen (253, 255, 259). The development of a vaccine that induces a mucosal response against both the historical and hypervirulent strains of *C. difficile* could potentially be effective in preventing primary and recurrent CDI, even in those most susceptible to poor outcomes (i.e. immunocompromised patients, those on antibiotic treatment and the elderly) (65, 286, 287). Recently, the Ward laboratory has developed a candidate mucosal vaccine based on an attenuated *Salmonella* vector (YS1646) that targets the RBDs of TcdA and TcdB (rbdA and rbdB, respectively) of a historical strain of *C. difficile*. When given using a multimodal schedule (i.e. a single i.m. injection on D0 and three p.o. doses of the *Salmonella*-based vaccine on D0, D2, D4), this novel strategy demonstrated 100% efficacy in a lethal challenge murine model (105). However, high strain-dependent variance in the RBD of TcdB suggested that the more conserved region of TcdB, the GTD, might provide broader protection, particularly against NAP1/B1/027 strains that are generally resistant to antibodies raised against the historical TcdB RBD (54-57). Our findings confirmed the immunogenicity of rGTD delivered i.m., and demonstrated high efficacy and immunogenicity of the *Salmonella*-based GTD + rbdB combination vaccine given multimodally. The next step would be to evaluate the mucosal response.

2.3.1 Optimizing NAP1/B1/027 laboratory culture

C. difficile, as its name suggests, is notoriously difficult to culture in laboratory settings and is extremely sensitive to oxygen (281-283). Oxygen concentrations >2% have major impact on *C. difficile* vegetative cell viability (288). Therefore, complete anaerobic conditions and

meticulous anaerobic techniques are required (281). When the current work was initiated, the protocol for *C. difficile* culture in our lab was satisfactory for a laboratory strain (VPI 10463) but needed to be optimized for NAP1/B1/027 culture since these strains proved to be even more sensitive to low oxygen concentrations (Table 1, Exp. #1) (105). Generally, *C. difficile* cultures are grown for ≥48h (11, 281-283). After much trial and error, the optimized protocol involved increasing the time of culture to 72h and the inoculum size (100 µL) yielding a consistent OD >1.000 (Table 1, Exp. #014-017). Why historical strains of *C. difficile* like VPI 10463 generally take only 24h to grow to high titers but NAP1/B1/027 takes 72h is currently unknown. One explanation could be strain-dependent differential gene expression (289). Weiss et al. used RNA-sequencing to study the transcriptional response of strain 630 and a NAP1/B1/027 strain (CD196) to an environment with 1.5% oxygen (289). They observed that 80 genes were differentially expressed in anaerobic conditions (i.e. basal level). These differentially-expressed genes included genes involved in sugar transport, and carbon and amino acid metabolism which, in turn, could affect the growth of these strains (289). Regarding the need for an increased inoculum size, Shida et al. studied the impact effect of inoculum size and nutrients on bacterial growth and showed that an increase in inoculum size could significantly reduce lag time (290). Such an effect in the NAP1/B1/027 strain we used could help to explain why increasing the inoculum size to 100 µL was necessary for optimal growth kinetics (290).

Since *C. difficile* is known to be extremely sensitive to low concentrations of oxygen, several approaches were explored in this work to decrease oxygen in the growth chamber. These included increasing the reduction time of the media and the concentration of the reducing agent, L-cysteine (Table 1, Exp. #7-8) (281, 291). The requirement for a second inoculation could potentially be explained by unappreciated exposure to oxygen at the time of the first inoculation (i.e. human error) (Table 1, Exp. #5), but the effect was not consistently seen. Adding the second inoculum to the protocol may simply have acted as an 'insurance policy' to mitigate against small breeches in technique. Although BHIS is widely used for *C. difficile* culture and several groups have suggested its superiority over meat broth, we showed that meat broth routinely promoted more growth of the NAP1/B1/027 strain we used than BHIS as evidenced by a higher OD value (1.453 vs. 1.213; Table 1, Exp. #2-3) (281, 292). Although these issues significantly delayed initiation of the later studies, this work demonstrated quite clearly that there is no 'one-size-fits-all' protocol for reliably growing different *C. difficile* strains to high density. This work also

demonstrates the experimental challenges that can be encountered when using clinical isolates like our NAP1/B1/027 strains in contrast to well-known and well-characterized 'laboratory strains' like VPI 10463.

2.3.2 Murine model for NAP1/B1/027 challenge

Once the NAP1/B1/027 strain could be reliably grown to high density, the next step was to modify the existing mouse model in the Ward laboratory for NAP1/B1/027 challenge. The reference model originally developed by Chen et al. had already been adapted slightly by Winter et al. from our lab for VPI 10463 challenge (104, 105). In both the Chen and Winter protocols, mice are given acetic acid in drinking water one week prior to antibiotic treatment and an i.p. dose of clindamycin 24h before infection to disrupt the gut microbiome and render the mice susceptible to *C. difficile* infection (104, 105). Again, some trial-and-error experiments were needed to show that, for NAP1/B1/027 challenge, the only modification that proved to be necessary was administration of the clindamycin 72h before infection instead of 24h. This modification was introduced as a practical matter since these experiments were done in parallel with optimization of NAP1/B1/027 culture. Because the reliable culture of NAP1/B1/027 took considerably more time than the historical strain, the timing of the challenge infection was delayed by 48h. This delay did not affect the susceptibility of the mice since we obtained an 80% mortality rate after NAP1/B1/027 challenge with the highest dose (3.06×10^3 CFU/mouse). Furthermore, Buffie et al. have previously shown that a single dose of clindamycin (200 µg i.p.) reduces the diversity of the gut microbiota of mice for at least 28 days so the timing of this last step in altering the gut microbiome is not likely to be critical (293). As a result, establishment of *C. difficile* in mice would most likely be possible for up to 28 days after clindamycin and our results were not particularly surprising.

Although we were able to establish a murine model for NAP1/B1/027 challenge and conduct initial studies, there are still some important limitations of this model. First, the challenge dose was estimated using the linear equation $y = 5.14 \times 10^5 X - 7.17 \times 10^4$, where X and y represent the OD and CFU/ml, respectively. Accurate assessment of the dose used in any given experiment (CFU/mouse) was therefore only obtained 24h after administration of the challenge infection (i.e. once the CFU counts of plated *C. difficile* could be assessed). Second, the maximum dose we were able to use for infection was relatively limited due to our inability to grow NAP1/B1/027 to an OD

higher than 1.059. As a result, the range of doses we assessed was limited (0 to 3.06 x 10^4 CFU/mouse) which likely had an impact on the maximum mortality observed in the control groups.

It is important to note that the extent of this 'preliminary' work was dictated by the fact that relatively few laboratories have established mouse NAP1/B1/027 challenge models (104). In fact, there are no well-characterized 'laboratory strains' of NAP1/B1/027 and the level of virulence of these different strains in mice is somewhat controversial. For example, Lanis et al. showed that TcdB of NAP1/B1/027 is 4-fold more pathogenic than a classical strain in mice (54). Orozco-Aguilar et al. used the murine ileal loop model to demonstrate that bacteria-free supernatant from NAP1/B1/027 induced the strongest pro-inflammatory response and that this correlated with increased cellular infiltration and epithelial damage (294). In contrast however, Chen et al. have suggested that a NAP1/B1/027 strain – BI17 in their study – may be less pathogenic than VPI 10463 (104). Although some further optimization will likely be required to fully exploit the NAP1/B1/027 challenge model established in the Ward laboratory through this work, we were nonetheless able to generate early proof-of-concept immunogenicity and efficacy results.

2.3.3 Immunogenicity of recombinant GTD

As noted in the introduction, almost all candidate vaccines, including those that have entered clinical trials, have targeted the RBD regions of the *C. difficile* toxins (105, 147, 253, 255, 257, 259, 295, 296). Despite its potential importance in defending against NAP1/B1/027 strains, remarkably little effort has been devoted to studying GTD-based vaccines and no GTD reagents (e.g. mono- or polyclonal antibodies) are commercially available to our knowledge (56, 262, 264). This is somewhat surprising since GTD has been previously shown to be highly immunogenic (57, 273, 297, 298). In fact, it has been suggested that GTD is the immunodominant portion of TcdB (i.e. the portion with the highest number of neutralizing epitopes) (57, 299).

To confirm the immunogenicity of rGTD designed in the Ward laboratory, we first demonstrated that mice given 3 doses of rGTD i.m. over a 5-week period elicited high TcdB-specific IgG titers (1.36 x 10^4 ng/mL) compared to PBS indicating that our rGTD was immunogenic. Although the ultimate goal of the work was the development of a vaccine that could elicit both systemic and mucosal anti-GTD immunity, classical i.m. prime-and-boost strategy was used for these initial experiments to demonstrate the effective priming of systemic immunity with subsequent boosting the immune response by subsequent doses (i.e. stronger, faster, higher quality

and durable response) (300). In our work, the two subsequent booster doses clearly increased IgG titers. It is noteworthy that the toxoid and fusion protein candidate vaccines developed by Pfizer and Sanofi Pasteur, and Valneva, respectively, have all used this approach and have all required 3 i.m. doses to induce a strong IgG response (253, 255, 257, 259).

One important limitation of this study is that the TcdB used to coat the ELISA plates in the IgG assay came from a historical strain, not a NAP1/B1/027 strain. This decision was driven by the unavailability of reference NAP1/B1/027 reagents and is true for all IgG ELISA results reported in this work. Two critical catalytic residues of GTD, D286 and D288, are conserved across all TcdB variants (301). However, the overall amino acid sequence identity of GTDs from different TcdB variants ranges between 79% and 100% (301). As a result, the rest of the NAP1/B1/027 GTD is unlikely to be identical to the GTD of a historical TcdB. Therefore, serum antibodies of the mice vaccinated with rGTD in our work (with the sequence obtained from a NAP1/B1/027 strain) may not recognize certain epitopes of historical GTD and the concentration of TcdB-specific IgG titers obtained may be under-estimated. Although we could have used our own rGTD to coat the ELISA plates, the clinical relevance of these results might have been questionable since rGTD is a small protein (62 kDa) compared to the full-length TcdB (270 kDa) and any effective anti-GTD immunity would have to recognize this domain in the whole toxin *in vivo* (302). Although, the optimal approach would be to use a full-length NAP1/B1/027 TcdB, such reagents were not available as noted above. Nevertheless, the IgG titers against the historical TcdB induced by the repeated injections of rGTD were high and increased with each dose. These data suggest that a combined anti-GTD and anti-rbdB, hereafter referred to as RBD, vaccine-induced response has the potential to act in either an additive or synergistic fashion against TcdBs from a broad range of clinical *C. difficile* isolates.

2.3.4 Proof-of-concept: Efficacy and immunogenicity of the RBD, ‘solo’ GTD and combined GTD + RBD vaccines against NAP1/B1/027

Having established the immunogenicity of our rGTD which was the same protein that would theoretically be delivered by our *Salmonella*-vectored oral vaccine candidate, the next step was to evaluate the efficacy of a classical RBD-targeting vaccine alone against a NAP1/B1/027 strain and the possible additive or synergistic effects of combining the anti-RBD and anti-GTD vaccines. These experiments used the standard, repeated i.m. dosing, as a positive control but

focused primarily on the prime-pull strategy demonstrated by Winter et al. to be effective with anti-RBD targeted vaccines (105). This latter strategy is intended to 'prime' the immune system with an i.m. dose of recombinant antigen to elicit antigen-specific systemic responses and then 'pull' the response towards the gastrointestinal tract with an oral vaccine to establish long-term local immunity (105, 303-306).

Somewhat surprisingly, a single dose of the i.m. RBD vaccine induced high levels of anti-TcdB IgG antibodies and provided 100% protection from lethal challenge with a NAP1/B1/027 strain suggesting significant cross-protection between the VPI 10463 and NAP1/B1/027 strains. Interestingly, multimodality vaccination with the same antigen (i.e. a single dose of the historic TcdB RBD on D0 and three p.o. doses of the same antigen using the YS1646 vector on D0, D2, D4) provided only 80% protection. This difference did not reach statistical significance however and is inconsistent with evidence on cross-protection in the literature. As noted above, Lanis et al. demonstrated that at least 11 epitopes in the RBD of TcdB differ between a historical and a NAP1/B1/027 strain, and that antiserum against NAP1/B1/027 RBD does not neutralize RBD from a typical strain (ribotype 003). Another study by Qiu et al. showed that anti-TcdB monoclonal antibodies targeting the RBD from the reference strain VPI 10463 do not neutralize TcdB from NAP1/B1/027 strains (307). It is possible that the i.m. dose used in this study and/or the relatively low inoculum we were able to use in the challenge model limited this model's ability to discriminate.

Much more promising was the fact that both 'solo' GTD-based vaccination and the combined GTD + RBD vaccination given multimodally had 100% efficacy against NAP1/B1/027. Furthermore, although this experiment was only performed once, the 'solo' GTD multimodal vaccine appeared to enhance the protective efficacy of the RBD multimodal vaccine when both were given together (Fig. 10A). Although the 'solo' GTD vaccination was as effective as the combined GTD + RBD vaccination when delivered using the multimodal vaccination, a difference could be seen in the IgG response induced by these vaccination strategies. The GTD + RBD multimodal vaccine induced higher TcdB-specific IgG titers than the 'solo' GTD multimodal vaccine (3.15×10^4 ng/mL vs 7.3×10^3 ng/mL, respectively) suggesting that GTD and RBD may work in an additive or synergistic way to enhance the humoral immune response generated. However, this would need to be confirmed with further studies that measure neutralizing titers as

well as, experiments to assess the mucosal (IgA titers) and cellular responses (Th1, Th17, and iTregs) (discussed in section 2.3.5.).

Acknowledging the limitations in comparing non-clinical and clinical observations, it may still be noteworthy that efficacy of 80% shown by the RBD multimodal vaccine is very high compared to the performance of the candidate i.m. vaccines from Pfizer (31%) and Sanofi Pasteur (-5.2%) in their failed Phase 3 studies (253, 259). These data raise the obvious question of whether or not targeting the TcdB RBD alone with a multimodality approach in humans could significantly improve protection across different strains (i.e. there would be no need for a combined GTD + RBD vaccine). Although this hypothesis may eventually need to be tested, we believe that both 'ends' of TcdB with a multimodal approach will protect against a wider range of circulating *C. difficile* strains than even multimodal vaccination against RBD alone. There are several reasons for this belief. First, most CDI cases are caused by historical strains whereas NAP1/B1/027 strains account for 22-36% of all CDI cases so a vaccine that offers high protection against both historical and hypervirulent strains is important (46-48, 308). Second, *in vitro* studies demonstrated that anti-TcdB monoclonal antibodies targeting the GTD from the reference strain VPI 10463 (CANmAbB1) was more potent in neutralizing hypervirulent NAP1/B1/027 strains than non-NAP1 strains, and anti-TcdB monoclonal antibodies targeting the RBD (CANmAbB4) from VPI 10463 was not able to neutralize TcdB of NAP1/B1/027 strains (307). This suggests that a combination of both would generate the most protective response against both non-NAP1 and NAP1/B1/027 strains. Third, Winter et al. showed that the RBD multimodal vaccine protects 100% of mice from VPI 10463 infection while our own data showed that this efficacy falls to 80% against NAP1/B1/027 (105). Our studies also demonstrated that the GTD multimodal vaccine not only offers 100% protection from death but also largely prevents the development of CDI-related symptoms following NAP1/B1/027 challenge. Together, these results support the suggestion that combination GTD + RBD multimodal vaccine may yield important advantages. Finally, GTD is conserved across all known *C. difficile* strains suggesting that combined GTD + RBD vaccination may offer some level of protection against potential newly emerging hypervirulent strains (e.g. ribotypes 078 and 023) (56, 57, 309, 310).

Although the multimodal schedule seems logistically complex, several studies have used a similar approach to prevent infections and treat cancers (304, 311, 312). Furthermore, the proposed

vaccine would require only three visits to a clinic since three i.m. doses are required and the three oral doses can be self-administered (e.g. similar to how the vaccine against *Salmonella enterica* serovar Typhi strain Ty21a (Vivotif) is used) (313). The requirement for three visits to a clinic for a vaccine is similar to several vaccines offered today (e.g. the hepatitis B vaccine) and, in the end, would be a small 'price' to pay for better protection (314). We believe that the first potential use of such a candidate GTD + RBD multimodal vaccine would be in individuals who have had primary CDI as prophylaxis for recurrence. Many of these individuals would likely still be in close contact with health-care settings when the series of vaccines would need to be given since CDI is still primarily nosocomial. This would facilitate vaccine compliance.

2.3.5 Limitations of our study

This study has several limitations beyond those already mentioned. First, a perfect animal model does not exist for the study of CDI. As mentioned previously, hamsters have been used since the discovery of *C. difficile* but the lack of hamster-specific reagents and genetically modified animals limits the use of this model (103). A mouse model was developed in 2008 by Chen et al. but the level of virulence of *C. difficile* and – more specifically of NAP1/B1/027 strains – in mice is controversial (104). In addition, mice are natural hosts of wild-type S. Tm and infected mice exhibit symptoms similar to typhoid fever caused by *Salmonella enterica* serovar Typhi in humans (315). Winter et al. observed S. Tm YS1646 colonization in the spleen and liver of mice for up to 1 to 2 weeks post-vaccination suggesting that these animals may have prolonged exposure to the *C. difficile* antigens compared to what may occur in humans (105). In humans, such dissemination of S. Tm is not expected since the YS1646 strain contains an *msbB* gene deletion rendering it highly sensitive to physiological carbon dioxide (CO_2) (316). Although this fact is likely to contribute to the safety of this vector, human studies will be required to determine whether or not exposure to *C. difficile* antigens delivered by the YS1646 vector will be 'long enough'. Furthermore, another limitation of using a murine model is that TcdA is more potent than TcdB in mice while the opposite is apparent in humans (2, 317-320). Alternative animal models include zebrafish, hares, rabbits, guinea pigs, prairie dogs, quails, foals, monkeys, Syrian hamsters and piglets (103). Only the latter two have been extensively used however. Hamsters, as mentioned previously, are extremely vulnerable to CDI such that only the most severe manifestations of this disease can be replicated in this model (321). Although pigs are a natural host for some *C. difficile*

strains that also infect humans (e.g. type 078), they are only susceptible to CDI in the first 2 weeks of life making multi-dose vaccine efficacy studies difficult (322, 323). The piglet model is also very expensive since these piglets need to be delivered by Caesarean section and maintained in germ-free conditions (324). Vaccine immunogenicity and efficacy studies are further limited by the lack of hamster- and piglet-specific reagents (103).

Second, the GTD + RBD multimodal vaccine that we studied targeted only TcdB while both TcdA and TcdB contribute to CDI in mice and humans (3, 317, 325, 326). We chose to focus our work on anti-TcdB responses because TcdB is generally believed to be more cytotoxic (i.e. more potent) than TcdA in humans (318, 319, 327-329). Leuzzi et al. showed that antibodies raised against TcdA cannot neutralize TcdB, and vice-versa (i.e. no cross-protection) (297). Although the GTD + RBD multimodal vaccine showed 100% efficacy, further development of the proposed vaccine may require addition of antigens from TcdA.

Third, our study lacks the use of cell-based assays to evaluate the immunogenicity of the RBD, GTD and GTD + RBD vaccines. Although animal models do not consistently predict immunogenicity in humans, cell-based immune assays in our model might improve the predictive accuracy of our work thus decreasing the use of animals and eventually increasing the efficiency of target choice (330-332). Therefore, in future experiments, we could analyze not only the antibody responses, but also peripheral blood mononuclear cells (PBMCs) cytokine and immune cell profiles using ELISA and flow cytometry (332-334). These assays would provide pertinent complimentary information since preclinical studies to assess safety, toxicity and efficacy of the vaccines are still required before translation to humans (334)

Last, our study did not include assessment of the RBD and GTD + RBD vaccine efficacy against other strains. Approximately 29% cases of severe CDI are caused by NAP1/B1/027 strains (310). However, types 023 and 078 represent 35% and 23% of severe CDI cases, respectively (310). Ribotype 023 is not a hypervirulent strain since it is not associated with higher mortality but disease severity is comparable to ribotypes 027 and 078 (310). Ribotype 078 (NAP7/8) infects primarily younger patients and is more frequently community-acquired (335). Therefore, to further assess the potential of the GTD + RBD vaccine as a 'universal' *C. difficile* vaccine, preclinical tests should be done to assess efficacy and immunogenicity against other *C. difficile* strains, most notably ribotypes 023 and 078.

2.3.6 Future directions

Our findings showcase the potential of a live-attenuated, YS1646-vectored mucosal vaccine against NAP1/B1/027. However, further studies are clearly needed to complete the non-clinical development of this vaccine.

First, it will be important to evaluate mucosal responses given their importance in the host defense against CDI (188, 233, 234, 239-242). For this purpose, measurement of IgA titers in the intestines (i.e. secretory IgA, sIgA) using Winter's procedure will be used instead of measuring serum IgA titers (105). The potency of polymeric secretory IgA against viral pathogens can be at least an order of magnitude higher than that of monovalent serum IgA or IgG (336). More relevant is the importance of sIgA in the intestinal lumen against enteric pathogens and toxins (337, 338).

Second, the crude levels of IgG and IgA titers do not give a full picture of their neutralizing capacity. In our work, serum IgG titers were higher in the GTD + RBD multimodal group compared to the 'solo' GTD multimodal group yet both vaccines had 100% efficacy against NAP1/B1/027 challenge. A toxin neutralizing assay could potentially be used to discriminate between these two formulations (287, 307, 329). This assay would include microscopic cytotoxicity analyses to determine whether or not the antibodies generated can prevent signs of cell death (i.e. cell rounding) (307).

Third, cellular responses to these vaccine candidates could be measured by flow cytometry on cells obtained from the spleen, inguinal and mesenteric lymph nodes, lamina propria and Peyer's patches (339-341). It would be of particular interest to analyze individual Tfh (CXCR5 and PD-1), Th1 (T-bet and IFN-γ), Th17 (ROR-γt and IL-17A) and iTreg (FoxP3) cells populations as well as the overall pattern of response (191, 340, 342, 343).

Finally, further development of this vaccine would need to proceed to chromosomal integration of the most promising antigenic sequences into YS1646 strains. Although this process would limit the copy number of the antigen genes, it would create a more stable vector suitable for use in humans (344). Antibiotic resistance to the YS1646 strains would be abolished, and the likelihood of random mutation and deletion of the antigenic DNA would be decreased (345).

CONCLUSION

Currently, there is no vaccine to protect against CDI in any population, including those at great risk of poor outcomes like the elderly, the debilitated and the immunocompromised (65). This project represents the first of many steps required for the development of a *C. difficile* vaccine potentially targeting both historical and NAP1/B1/027 strains. We showed that a combination vaccine comprised of both the GTD and RBD from TcdB administered multimodally has 100% efficacy, prevents CDI symptoms and induces a protective IgG response against NAP1/B1/027 challenge. Although the vaccination schedules we tested were somewhat complex (i.e. 2 different i.m. formulations and 2 different oral formulations delivered over almost 6 weeks), this work demonstrated clear proof-of-concept for the idea targeting both the TcdB receptor-binding and glucosyltransferase domains to broaden protection against *C. difficile*. Future work will seek to simplify this schedule by combining the i.m. antigens and generating YS1646 strains capable of delivering both the RBD and GTD antigens. Unfortunately, full evaluation of the mucosal response generated by the candidate vaccines has yet to be completed but previous work in the Ward laboratory with several YS1646-based vaccines has shown that this vector can reliably generate antigen-specific IgA responses in the intestinal tissues (105, 278). Nevertheless, this research brings us one step closer to developing a potential universal *C. difficile* vaccine.

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