Preclinical development of a novel vaccine targeting *Clostridioides difficile* using an attenuated *Salmonella* Typhimurium vector

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For Dorothy and Ruth
1920-2018 and 1938-2018

Thank you for your
encouragement and love

You are missed

ABSTRACT

Clostridioides difficile infection (CDI) is one of the most important nosocomial infections in the world. In 2012, approximately 40,000 CDI cases were reported in Canada with a cost of ~$300 million. In the last decade, the rate of hospital-acquired CDI cases has decreased, however community-acquired CDI rates are increasing. CDI is usually initiated by antibiotic use that disrupts the normal gastrointestinal microflora leading to *C. difficile* overgrowth and the production of two major toxins A and B (TcdA and TcdB) that cause CDI-associated pathology. Reducing morbidity and mortality from CDI requires the development of new tools, such as vaccines. While a small number of candidate vaccines targeting *C. difficile* have entered clinical trials, none has targeted the gut mucosa. Among the many potential advantages of live *Salmonella*-based vaccines is their ability to deliver antigens to mucosal surfaces in the context of regional bacterial 'invasion' – a scenario likely to elicit strong humoral and cellular immune responses at the site of *C. difficile*-induced pathology. We used an attenuated strain of *Salmonella* Typhimurium, YS1646 as a vector to develop a candidate vaccine targeting the highly immunogenic C-terminal receptor binding domains (RBD) of TcdA and TcdB *of C. difficile*. Anti-RBD antibodies have been shown to neutralize the corresponding toxins and protect against *C. difficile* challenge in animal models. To address our first aim of making a vaccine that would stimulate both mucosal and humoral immunity, we generated candidate YS1646 strains bearing plasmids that express and secrete the RBDs of TcdA or TcdB. Our best candidates elicited both systemic and mucosal antibody responses in C57BL/6 mice when given in a multimodality schedule: ie: one dose of recombinant protein intramuscularly (IM) plus 3 doses of the YS1646 candidates orally (PO) over one week. Two of our constructs were tested in a *C. difficile* challenge mouse model and achieved 100% protection (versus 30% survival in the control group). The PO vaccines alone gave ~80% protection. For our second aim, we examined the longevity of the responses elicited by our vaccine candidates. IgG (serum) and IgA (gut) titers elicited by multimodal vaccination were maintained up to 6 months after vaccination. Multimodal vaccination significantly protected mice that were challenged 6 months after vaccination (83-100% survival versus 33% in PBS controls). PO vaccines alone gave ~90% protection. Our third aim was to develop stable vaccine candidates without a mobile genetic element. To do this, we established 6 YS1646 strains with stable chromosomal expression of the targeted RBD antigens. After *in vitro* screening, we selected two

candidates to move forward. When delivered in a multimodal vaccination schedule, these candidates generated IgG titers, with a skewing towards the IgG1 subtype and an increase in antigen specific IL-5 production in the mesenteric lymph node 32 days after vaccination. Oral delivery alone elicited a bias towards IgG2c antibodies and increased antigen-specific GM-CSF production in the Peyer's patches. Upon challenge with a clinical *C. difficile* isolate, mice that received multimodal vaccination against both toxins had 94% survival (versus 38% survival in the PBS control group). Oral vaccination against TcdA at a higher dose elicited 100% survival (versus 30% in the control group). In summary, we have developed an orally-delivered vaccine candidates that elicit both systemic and mucosal immune responses in mice and are highly protective against *C. difficile* infection. Through this project, we have gained considerable insight into the immunological interactions between the host and *S.* Typhimurium YS1646-based vaccines and valuable information for the rational design of the first in human study of these novel vaccine candidates.

RÉSUMÉ

L'infection de *Clostridioides difficile* (ICD) est l'une des infections nosocomiales les plus importantes. En 2012, environ 40 000 cas d'ICD ont été rapportés au Canada. L'ICD est généralement déclenchée par l'utilisation d'antibiotiques qui perturbent le microbiome gastro-intestinal normal, entraînant une prolifération de *C. difficile* et la production de deux toxines majeures A et B (TcdA et TcdB) qui provoquent la pathologie associée à l'ICD. Pour réduire la morbidité et de la mortalité de l'ICD, le développement de nouveaux outils, tels que les vaccins, sont nécessaires. Alors qu'un petit nombre de vaccins candidats ciblant *C. difficile* sont entrés dans les essais cliniques, aucun n'a ciblé la muqueuse intestinale. Parmi les avantages des vecteurs de vaccins de *Salmonelle*, ils ont la capacité de livrer des antigènes aux surfaces muqueuses dans le contexte d'une « invasion » bactérienne régionale, un scénario susceptible de provoquer des réponses immunitaires au site où *C. difficile* induit la pathologie. Nous avons utilisé une souche atténuée de Salmonelle Typhimurium, YS1646, comme vecteur pour développer un vaccin candidat ciblant les domaines de fixation du récepteur (FDR) hautement immunogènes de TcdA et TcdB de *C. difficile*. Il a été démontré que les anticorps (ac) spécifiques pour FDR neutralisent les toxines correspondantes et protègent contre l'infection par *C. difficile* dans des modèles animaux. Pour notre premier objectif, nous avons généré des souches candidates YS1646 portant des plasmides qui expriment les FDR de TcdA ou TcdB. Nos meilleurs candidats ont provoqué des réponses ac systémiques et muqueuses chez les souris lorsqu'ils ont été administrés selon un schéma multimodal, c'est-à-dire : une dose de protéine recombinante par voie intramusculaire (IM) et trois doses des candidats YS1646 par voie orale (PO) sur une période d'une semaine. Quand les vaccins PO seuls ont été testées dans un modèle de souris avec provocation de *C. difficile* ils ont obtenu une protection d'environ 80% (contre 30% de survie dans le groupe placebo de PBS (GP)). Pour notre deuxième objectif, nous avons examiné la longévité des réponses suscitées par nos candidats vaccinaux. Les titres d'IgG (sérum) et d'IgA (intestin) induits par la vaccination multimodale ont été maintenus jusqu'à six mois après la vaccination. La vaccination PO seul a protégé de manière significative les souris qui ont été infectées six mois après la vaccination (survie de 90 à 100% contre 33% chez le GP). Notre troisième objectif était de développer des candidats vaccinaux stables sans élément génétique mobile. Pour ce faire, nous avons établi six souches YS1646 avec une expression

chromosomique stable des antigènes FDR. Lorsqu'ils deux candidats sont administrés selon un schéma de vaccination multimodale, ils génèrent des titres d'IgG, avec une inclinaison vers le sous-type IgG1 et une augmentation de la production d'IL-5 spécifique de l'antigène dans le ganglion lymphatique mésentérique 32 jours après la vaccination. L'administration orale seule a provoqué une inclinaison en faveur des ac IgG2c et une production accrue de GM-CSF spécifique de l'antigène dans les plaques de Peyer. Après l'infection de *C. difficile*, 94% des souris qui ont reçu une vaccination multimodale contre les deux toxines ont survécu (contre 38% de survie dans le GP). La vaccination orale contre le TcdA à dose plus élevée a induit 100% de survie (contre 30% dans le GP). En résumé, nous avons développé un vaccin candidat administré par voie orale qui provoque des réponses immunitaires systémiques et muqueuses chez les souris et qui est hautement protecteur contre l'infection de *C. difficile*. Grâce à ce projet, nous avons acquis des connaissances considérables sur les interactions immunologiques entre l'hôte et les vecteurs de vaccins de *S.* Typhimurium YS1646 et des informations précieuses pour la conception rationnelle de la première étude humaine de ces nouveaux vaccins candidats.

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LIST OF ABBREVIATIONS

aa	Amino acids
ADL	Activities of daily living
alum	Aluminum hydroxide gel
AMR	Antimicrobial resistance
APC	Antigen presenting cell
aroC	Chorismate synthase
asd	Aspartate-semialdehyde dehydrogenase
ATCC	American Type Culture Collection
BCR	B cell receptor
BHIS	Brain heart infusion
BMDC	Bone marrow-derived dendritic cell
^{o}C	Degree Celsius
CA	California
CA-CDI	Community acquired *C. difficile* infection
CAT	Catalytic domain
CBD	CDTb binding domain
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CDI	*Clostridioides difficile* infection
CDT	*C. difficile* transferase toxin or binary toxin
cfu	Colony forming units
CI	Chromsomal Integration
CIHR	Canadian Institutes of Health Research
cm	Centimeter
CMC	Chemistry-Manufacturing-Control
CO_2	Carbon Dioxide
COVID-19	Coronavirus disease 2019
CpG	Cytosine–phosphate–guanine
CPD	Cysteine protease domain
CRISPR	Clustered regularly interspaced short palindromic repeats
CROP	Combined repetitive oligopeptides
CS	Clinical Score
CSPG	Chondroitin sulfate proteoglycan
CTD	Central translocation domain
Cwp	Cell wall protein
CXCL	C-X-C motif chemokine ligand
DAP	diaminopimelic acid
DAPI	4′,6-diamidino-2-phenylindole

DC	Dendritic cell
DFAT	L-Glutamine:D-fructose-6-phosphate aminotransferase
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dpi	Days post infection
EDTA	Ethylenediaminetetraacetic acid
EF	Extrafollicular site
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-linked immuno absorbant spot
et al	et alia
FBS	Fetal Bovine Serum
Fig	Figure
FliC	Flagellin
FliD	Cap Protein
Flp	Recombinase flippase
FRT	Flippase recombination target
FZD	Wnt Receptor Frizzled
GALT	Gut-associated lymphoid tissue
GC	Germinal Center
glms	Glutamine-fructose-6-phosphate aminotransferase
GLP	Good laboratory practices
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMMA	Generalized modules of membrane antigen
GMP	Good manufacturing practices
GRO-α	Growth-related oncogene α
GSK	GlaxoSmithKline
GTD	Glucosyltransferase domain
h	Hours
H_2SO_4	Sulfuric Acid
HA-CDI	Health care-acquired CDI
HBSS	Hanks’ balanced salt solution
HCW	Health care worker
his	Histidine
HMW	High molecular weight
HRP	Horseradish peroxidase
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin A

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IL	Interleukin
ILC	Innate lymphoid cell
IM	Intramuscular
iNOS	Inducible Nitric oxide synthase
IP	Intraperitoneal
IPTG	Isopropyl-β-d-1-thiogalactopyranoside
IR	Intrarectal
IV	Intravenous
kDa	kilodalton
kV	kilovolts
lac	Lactase
LB	Luria Broth
LDS	Lithium dodecyl sulfate
LMW	Low molecular weight
LP	Lamina propria
LPL	Lamina propria lymphocyte
LPS	Lipopolysaccharide
LSR	Lipolysis-stimulated lipoprotein receptor
LTCF	Long term care facility
M	Molar
MA	Massachusetts
MAIT	Mucosa-associated invariant T
MCP-1	Monocyte chemo-attractant protein 1
MDDC	Monocyte derived dendritic cell
mg	milligram
MHC	Major histocompatibility complex
min	Minutes
ml	milliliter
mLN	Mesenteric lymph node
mM	milliMolar
MO	Missouri
MOI	Multiplicity of infection
MPD	Membrane and pore formation domain
mRNA	Messenger ribonucleic acid
msbB	Lipid A biosynthesis myristoyltransferase
MyD88	Myeloid differentiation primary response protein
NAP1	North American pulsed-field gel electrophoresis type 1

NET	Neutrophil extracellular traps
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
Ni-NTA	Nickel-Nitrilotriacetic Acid
nirB	Nitrite reductase B
NK	Natural Killer
nm	nanometer
NOD1	Nucleotide-binding oligomerization domain protein family 1
ns	Not significant
NTS	Non-typhoidal *Salmonella*
OD	Optical density
OMV	Outer membrane vesicle
ON	Ontario
OR	Oregon
pagC	phoP activated gene C
PaLoc	Pathogenicity locus
PAMP	Pathogen associated molecular pattern
PBS	Phosphate Buffered Saline
PC	Plasma cell
PCR	Polymerase Chain Reaction
PD-L1	Programmed cell death ligand 1
pH	Potential of Hydrogen
PhoP	Transcriptional regulatory protein
PhoQ	Sensor protein
PI	Protease Inhibitor
PO	Per os/oral
PP	Peyer's Patches
PRR	Pattern recognition receptor
PS	Polysaccharide
purI	Phosphoribosylformylglycinamidine synthase
QC	Quebec
RAR	Retinoic acid receptor
RBD	Receptor binding domain
rbdA	RBD of TcdA
rbdB	RBD of TcdB
RI-MUHC	McGill University Health Center Research Institute
RORγ	RAR-related orphan receptor γ
ROS	Reactive oxygen species
rpm	Revolutions per minute

RPMI	Gibco Roswell Park Memorial Institute
RpoS	RNA polymerase sigma factor
rrbdA	Recombinant RBD of TcdA
rrbdB	Recombinant RBD of TcdB
RT078	*C. difficile* ribotype 078
SCFA	Short Chain Fatty Acid
SCV	*Salmonella* containing vacuole
SD	Standard Deviation
SEM	Standard Error of the mean
SLP	S-layer protein
SopE2	*Salmonella* outer protein E2
SPI	*Salmonella* pathogenicity island
SptP	Secreted effector protein
ssaV	Secretion system apparatus protein
SseJ	*Salmonella*-translocated effector J
SspH	*Salmonella* secreted protein H
STAT	Signal transducer and activator of transcription
Ste	*Salmonella* translocated effector
Supp	Supplemental
T3SS	Type 3 secretion system
Tbet	T-box expressed in T cells
TcdA	*C. difficile* Toxin A
TcdB	*C. difficile* Toxin B
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TMB	3,3′,5,5′-tetramethylbenzidine
TNF	Tumour Necrosis Factor
Treg	Regulatory T cell
UK	United Kingdom
US	United States
USD	US Dollar
UV	Ultraviolet radiation
VA	Virginia
vol/vol	Volume by volume
wbc	White blood cell
WT	Wild type
wt/vol	Weight by volume
x g	Times gravity
µF	microfarad

µg	microgram
µl	microliter

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The work presented in this thesis contributes original knowledge to the fields of *Clostridioides difficile* vaccine design and the use of *Salmonella enterica* serovar Typhimurium as a vaccine vector. The specific contributions are as follows:

1. We developed *S.* Typhimurium YS1646 vaccine candidates that expressed a portion of the receptor binding domain of Toxins A and B through a plasmid-based expression system.
2. We demonstrated that when delivered in a multimodal strategy with recombinant protein delivered intramuscularly (IM), our vaccine candidates were immunogenic and protected mice from *C. difficile* challenge.
3. We demonstrated that the protective responses elicited by our vaccine candidates (both multimodal and antigen-expressing YS1646 alone) were maintained for 6 months after vaccination.
4. We demonstrated the oral delivery of the antigen-expressing YS1646 increases the splenocyte response to antigen stimulation 6 months after vaccination compared to IM delivery of recombinant antigen alone.
5. We developed antigen-expressing YS1646 candidates that had the antigen sequence chromosomally integrated (CI). These strains do not contain mobile genetic elements nor antibiotic resistance.
6. We demonstrated that the CI strains elicit both systemic and mucosal responses that protect mice from *C. difficile* challenge.

Taken together, we have developed vaccine candidates against *Clostridioides difficile* that elicit systemic and mucosal responses. Our candidates are suitable for further testing in efforts to begin clinical trials.

CONTRIBUTIONS OF AUTHORS

The candidate has chosen to present a manuscript-based thesis. This thesis contains three original manuscripts and is in accordance with the "Guidelines for Thesis Preparation" provided by the Faculty of Graduate and Postdoctoral Studies of McGill University. The candidate, Kaitlin Winter, is recognized as the principal author and to have performed the majority of the work of the manuscripts presented. The specific contributions of authors are as follows:

Chapter 1

Section 1.10 contains extracts from **Winter K**, Hassan AS, Dozois CM, Ndao M, Ward BJ. Current applications of *Salmonella enterica* serovar Typhimurium as a vaccine vector. Manuscript prepared for submission to *Frontiers Immunology*.

KW and AH wrote the manuscript and contributed equally. All extracts presented in this thesis were written by KW. BJW, CMD, and MN edited the manuscript.

The remainder of the literature review was written by KW and edited by BJW.

Chapter 2

Winter K, Xing L, Kassardjian A, Ward BJ. 2019. Vaccination against *Clostridium difficile* by use of an attenuated *Salmonella enterica* serovar Typhimurium vector (YS1646) protects mice from lethal challenge. Infect Immun 87:e00089-19.

This manuscript is reprinted from the journal *Infection and Immunity*. KW, LX, and BJW designed the study and experiments. LX and KW designed, constructed, and validated the plasmids. KW performed the animal experiments. AK performed experiments under the supervision of KW. KW and BJW performed analysis of the data and prepared the manuscript.

Chapter 3

Winter K, Ward BJ. Attenuated *Salmonella* Typhimurium vectored vaccine provides long-term protection against lethal *Clostridioides difficile* challenge. Manuscript prepared for submission to the journal *Vaccine* as a brief communication.

All authors contributed to the study and experimental design. KW performed all experiments. KW and BJW performed the analysis of the data and prepared the manuscript.

Chapter 4

Winter K, Houle S, Dozois CM, Ward BJ. Vaccination with a chromosomally integrated *Salmonella* Typhimurium vector protects mice from lethal *Clostridioides difficile* challenge. Manuscript prepared for submission to *npj Vaccines*.

All authors contributed to the study and experimental design, with SH and CMD lending their considerable expertise to the bacterial genetics experiments. KW and SH performed the chromosomal integration. KW performed all animal experiments. KW and BJW performed the analysis of the data and prepared the manuscript. All authors contributed to editing the manuscript.

Chapter 5

The general discussion was written by KW and edited by BJW.

Chapter 1: Literature review and research objectives

1.1 *CLOSTRIDIOIDES DIFFICILE*

Clostridioides difficile is gram positive, rod-shaped bacterium that was originally isolated in 1935 by Hall and O'Toole (1). It is an obligate anaerobe, that forms spores that are ubiquitous in the environment (2). Its species name – *difficile* - was given to it based on the difficulty early investigators encountered in trying to isolate it and maintain it in culture (3). It was first associated with human disease in 1978 and is the pathogen responsible for the majority of cases of antibiotic-associated diarrhea (4, 5).

1.1.1 Pathogenesis

In humans, *C. difficile* colonizes primarily the colon (6). It can cause a range of symptoms, from asymptomatic colonization to diarrhea to severe colonic inflammation leading to death (3). Severe *C. difficile* infection (CDI) is characterized by diarrhea, plaque formation in the colon, neutrophil influx in the lumen and pseudomembranous colitis. In some CDI cases, toxic megacolon and fulminant colitis can also occur (7). These severe symptoms can be very difficult to treat and are life-threatening (8). Even after successful treatment, 25-35% of patients will experience a recurrent infection within 60 days of the initial CDI (9).

1.1.2 Epidemiology

The risk of acquiring CDI increases with age and both antibiotic and proton pump inhibitor use (10, 11). The highest risk period of CDI is during antibiotic use and in the first month after cessation (3, 12). Clindamycin, cephalosporins and fluoroquinolones are antimicrobials that pose the highest risk for CDI. Macrolides and sulfonamides have also been associated with CDI, but to a lesser extent (12). Even when controlling for additional risk factors, increasing age is associated with CDI. One study estimated that every year above the age of 18 increases CDI risk by ~2% (10). 60% of CDI cases in 2011 in the United States (US) occurred in patients over the age of 65 (13). Functional status of patients over the age of 50 is also an independent risk factor for severe CDI (14). With the elderly population expected to double in

the US by 2050, it is likely that CDI will continue to be a major public health threat for the foreseeable future unless new approaches to its prevention and treatment are developed (13).

CDI rates in North America steadily increased until the late 2000s. At that time, the US reported ~500 000 infections, with 30 000 deaths annually (15). At that time, the burden of CDI was estimated to cost 6 billion USD/year. Approximately 30% of CDI cases were community-acquired (CA-CDI) (9, 16, 17). In 2012, there were 37 900 CDI cases in Canada, with an estimated cost of 281 million CAD (18). Due to increased decontamination procedures implemented in clinical settings, nosocomial or health care-acquired CDI (HA-CDI) rates have been decreasing since the late 2000s (19-22). HA-CDI rates in Canada in 2015-2017 are estimated to be 2.3-5.3/10 000 patient days, depending on the province and prevalence of the NAP1/B1/027 (NAP1) strain (19, 20). The NAP1 strain was initially identified as hypervirulent during a CDI outbreak in Quebec hospitals in 2003 (23). The increased virulence of NAP1 is associated with several changes in toxin regulation and function (24-26). Increased proportion of NAP1 cases are correlated with higher rates of CDI in hospitals, and NAP1 is the predominant strain of *C. difficile* identified in emergency departments of Canadian hospitals (19, 21). On a broader level, NAP1 is responsible for ~30% of CDI cases although the proportion of cases caused by NAP1 strains can be quite variable by year and by geography (24, 27). While HA-CDI rates are decreasing, CA-CDI rates have continued to increase since the late 2000s (20, 28). CA-CDI rates in 2017 in Canada were estimated to be 10.57-40.81/100 000 population (20).

1.1.3 Transmission

As most cases of CDI are acquired in a health care setting, the main reservoir for *C. difficile* is infected patients with contributions from contaminated health care workers and the hospital environment (29). *C. difficile* spores, which are discussed in more detail in **Section 1.1.6**, are the vehicle of transmission (30). Resistance of spores to bleach-free disinfectants can allow the rapid spread of *C. difficile* through health care facilities on contaminated equipment and staff (31-33). Outside of hospitals, long-term care facilities (LTCFs) are a major source of HA-CDI (34). A recent study in Alberta, concluded that 95% of CDI cases in LTCF residents were acquired in a LTCF (34).

With increasing CA-CDI rates, other sources of *C. difficile* transmission need to be considered. *C. difficile* spores are ubiquitous in the environment (35). *C. difficile* can colonize a wide range of vertebrate hosts from household pets, to poultry and other farm animals, to more exotic animals such as elephants and Kodiak bears (36). Among household pets, 10-50% of dogs and 20% of cats carry *C. difficile* (37). While no direct zoonotic transmission has been demonstrated yet, it may be possible. A recent study in North America, Europe and Australia observed shared strains with recent evolutionary history between animals (household and farm animals) and humans (38). A study in the Netherlands found that isolates of *C. difficile* ribotype 078 (RT078) recovered from hospitalised humans correspond with samples taken from pigs (39). These data correspond with those of a European study that found that the RT078 genome clusters geographically with extensive co-clustering between humans and animals across 22 countries (40). The prevalence of *C. difficile* spores in farm animals and the environment (and therefore root vegetables) and their genomic similarity to human isolates, has led to an active debate of whether or not CDI can be transmitted by food (41-43). Currently however, there is no direct evidence of foodborne transmission.

The role of asymptomatic carriers in transmission of CDI is also a subject of debate. Although very few healthy adults are asymptomatic carriers of CDI, several studies have estimated the rate of carriage to be 0-17.5%, with 1-5% carrying toxigenic strains (44-48). However, around 35% of babies and young infants are colonized with *C. difficile* (49). In the United Kingdom (UK), 13% of strains isolated from infants under the age of 2 were linked to CDI cases that were active at the time of sampling (50). Another study in the UK found that only 40% of HA-CDI cases were linked to another symptomatic case, with 45% of cases being genetically dissimilar to all other cases in the facility (51). In the US, one group linked 30% of HA-CDI cases to an asymptomatic carrier (52). These studies suggest that asymptomatic carriers can act as a reservoir for CDI. In a study focused on controlling this reservoir, active screening for *C. difficile* colonization upon hospital admittance reduced HA-CDI cases by 60% (53).

1.1.4 Cell structure

Clostridioides difficile is a gram positive, rod-shaped, spore-forming bacterium. Both flagellar and non-flagellar strains of *C. difficile* have been described. Non-flagellated strains are

less adherent to the intestinal mucosa (54, 55). Flagellin and the cap protein on the flagella, FliC and FliD respectively, are able to bind to murine mucus and Caco-2 cells, suggesting they play a role in colonization in flagellated strains (56). In some strains, the expression of the flagella is co-regulated with toxin expression and can act in tandem to elicit a stronger immune response from the host (57). The surface structure of *C. difficile* is mainly made up of two S-layer proteins (SLPs) (58). Low molecular weight (LMW) -SLPs are highly variable between strains, while high molecular weight (HMW) -SLPs are conserved between strains (59, 60). While both SLPs are involved in cell adherence, LMW-SLPs are surface exposed and HMW-SLPs are anchored in the cell wall and contribute to adherence to intestinal tissue and extracellular matrix proteins (61). Most SLPs are generated as one large molecule, SlpA, and then are cleaved at the cell surface. Cwp84 is a cysteine protease whose N-terminal domain cleaves SlpA into LMW-SLPs and HMW-SLPs. Cwp84 is conserved across strains, is immunogenic and plays a role in degrading extracellular matrix allowing *C. difficile* to spread more easily. The largest member of the CWP family in some strains of *C. difficile* is CwpV (62). It has 3 main domains and is cleaved into two fragments independently of Cwp84 (63). The N-terminal domain is highly conserved across strains and anchors the protein to the cell wall. The middle domain contains a serine-glycine rich linker sequence that is conserved across strains but has unknown functions. Finally, on the C-terminal domain, are 9 repeats of 120 amino acids (aa) that are highly immunogenic. Reynolds et al identified 5 antigenically distinct sequences for this domain across 31 strains of *C. difficile*. CwpV has phase variable expression controlled by the *C. difficile recV* gene and is only expressed in monomicrobial culture in 0.1%-10% of cells, depending on the strain. However, it appears to allow auto-aggregation, as high expression of CwpV leads to a change in colony morphology. *C. difficile* also produces polysaccharides, PS-I and PS-II. PS-II is common to all strains of *C. difficile* (64).

1.1.5 Spores

Spore formation is essential to *Clostridioides difficile* transmission (30). Vegetative cells are unable to survive in aerobic environments, thus the bacteria require metabolically dormant and highly resistant spores to continue the infectious cycle in new hosts (31). *C. difficile* spores can persist for more than 12 months in dry, inanimate environments and are resistant to bleach-

free disinfectants, antibiotics, and the host immune system (32, 65). They are implicated in 20-25% of recurrent infections.

Sporulation of *C. difficile* differs greatly from other highly studied spore-forming bacteria, such as *B. subtilis* and *C. perfringens* (66). Spo0A is the master transcriptional regulator that controls spore formation (67). Its activation leads to the activation of sporulation-specific RNA polymerase sigma factors that activate the sporulation pathway (68). The signals that trigger sporulation in *C. difficile* are unknown, but quorum sensing, nutrient starvation or other stress factors trigger sporulation in other bacteria. The *C. difficile* spore has several features to increase its dormancy, longevity, and resistance to the environment. In the core, DNA is bound by specialised DNA binding proteins that aid in UV resistance, and the cytosol is partially dehydrated (Fig 1.1A) (69). The core is surrounded by a peptidoglycan layer, the cortex. The cortex prevents additional water from entering the core to maintain dormancy and protects from extreme temperatures and ethanol-based sanitizers (70). The cortex is covered by several proteinaceous shells that form the coat (71). The coat protects the spore from oxidative insults and enzymatic digestion (72). The final outer layer is the exosporium, which aids in the binding of surfaces and intestinal epithelial cells (IECs) (Fig 1.1B) (73). One of the main components of the exosporium, BclA1 is poorly immunogenic in goats (73).

C. difficile germination occurs after ingestion of spores, upon their arrival in the small intestine (35). The area of the small intestine with the highest rates of spore germination is the ileum, likely due to the presence of germinants and a higher pH than the duodenum (74). Several primary bile acids which are secreted into the duodenum, act as germinants for *C. difficile* (75) and several vertebrate bile acids appear to be particularly effective including taurocholate, and deoxycholate as well as other cholates (76). Among the common human bile acids, taurocholate appears to be the most potent germinant (77, 78). In healthy patients, a normal microflora metabolizes bile acids into secondary bile acids, many of which are toxic to *C. difficile* (79). Antibiotic use decreases the population of bile acid-converting bacteria in the microflora, increasing the ease of germination for *C. difficile* spores (80). While several secondary bile acids act as inhibitors of *C. difficile* germination or growth, it would be an oversimplification to state that primary bile acids induce germination while secondary bile acids inhibit. For example,

chenodeoxycholate, a primary bile acid, and its derivatives inhibit germination. Although deoxycholates are germinants, they can also prevent *C. difficile* growth after germination (77, 78). *In vitro, C. difficile* spores require the presence of a co-germinant to begin germination. There are two main classes of co-germinants: calcium ions or amino acids, with glycine being the most potent activator of taurocholate-induced germination (81). When spore germination is induced, the spore releases Ca_2^+-dipicolinic acid, the core is rehydrated, the cortex degrades and finally, a vegetative cell emerges from the spore (82).

1.2 ROLE OF THE MICROBIOME IN *CLOSTRIDIOIDES DIFFICILE* INFECTION

As an extracellular pathogen living in the gastrointestinal tract, *C. difficile* interacts with the gut microbiome throughout its lifecycle. A disruption of the microbiome is necessary for *C. difficile* to successfully colonize the colon (83). The colonization resistance of the healthy human microbiome is hypothesized to occur via a competition for nutrients, ecological competition, and niche exclusion (84). Studies in both adults and the elderly have negatively correlated colonization with *Bacteroides* and *Bifidobacterium* species with CDI risk (85-88). *C. difficile* and the microbiome are able to interact through molecule secretion and metabolism in the gut. As noted above, some bile acids can act as germinants for *C. difficile* spores, and their metabolism is affected by the microbiome (75, 79). For example, antibiotic treatments that alter the microbiome and elevate bile acid levels in the cecum of mice actively promote germination (89, 90). Indole is a signalling molecule produced by several phyla in the gut microbiota, but not by *C. difficile* (91). In the healthy human gastrointestinal tract, indole is capable of modulating inflammation, enhancing barrier function and decreasing tight junction permeability (92-95). However, patients with CDI have higher levels of indole in their stool, compared to patients with non-CDI diarrhea (96). Through the quorum signalling peptide, Agr1, *C. difficile* induces increased indole production in other gut microbes (96). Indole can also inhibit the growth of some competitors in the gut microbiome, such as *Bacteroides* species (96).

Many of the risk factors for CDI are linked with changes in the microbiome (97). Antibiotic treatment decreases the diversity and can change the composition of the microbiome (98-100). Proton pump inhibitors increase the pH of the stomach, which can lead to changes in the gut microbiome (101, 102). Microbiome changes specific to the elderly will be discussed in

Section 1.5.2. Inflammation in the gut has also been associated with increased risk for CDI (103, 104). This could be due to the increase of antimicrobial peptides secreted into the gut during inflammation, which would limit the growth of otherwise protective gut microbes (105, 106). Indeed, patients with inflammatory bowel disease have a decreased diversity of Firmicutes and Bacteroidetes (107).

Finally, the gut microbiome has extensive points of possible interaction with the mucosal immune system, meaning changes to the microbiome could impact the response to CDI. For example, microbiota diversity has been demonstrated to strongly affect the diversity of IgA in the intestine (108). Several microbes in the microbiome are known to have an impact on T cell subsets in the intestine, such as *Bacteroides* species inducing colonic T regulatory cells (109, 110). Unsurprisingly, the relationship may go both ways, as mucosal immune responses may affect the intestinal microbiome. The intestinal microbiome of patients with active diarrhea, whether it is caused by CDI or not, are very similar, suggesting that either the mucosal immune response to a diarrhoeal disease or diarrhea impacts intestinal microbiome diversity (87).

1.3 *CLOSTRIDIOIDES DIFFICILE* TOXINS

Clostridioides difficile infection is a toxin-mediated disease. Most pathogenic strains of the bacteria produce two main toxins, Toxin A and Toxin B (TcdA and TcdB). Both toxins are glucosyltransferases that are single-subunit polypeptides. They share 48% aa sequence homology (111). 5-30% of strains also produce a binary toxin (CDT) (112), which will be discussed later in this section.

1.3.1 Toxins A and B

The genes for TcdA and TcdB are contained in the pathogenicity locus (PaLoc) (113-115). The PaLoc also encodes negative (tcdC) and positive (tcdR) regulators and the gene for a holing-like pore forming protein (tcdE) (116-120). The NAP1 strain has a deletion in the tcdC region, which may contribute to the strains' high toxin production (121). Toxin production is regulated by environmental signals. It is inhibited by the presence of glucose, amino acids, butanol and biotin. Short chain fatty acids (SCFAs), such as butyrate, and 37^{o}C in the environment activate toxin expression and secretion (122-124). Several isoforms of both toxins

have been described (125). As *C. difficile* is an extracellular pathogen and the toxins act in the cytosol, they must undergo several steps to reach their target. After secretion by the bacteria, TcdA and TcdB bind the target cells, are endocytosed, translocate across the endosomal membrane, are processed to release the biologically active toxin moiety, and finally modify their target proteins in the host cells.

1.3.1.1 Domain structure of toxins A and B

Both TcdA and TcdB have similar structures with 4 domains (Fig 1.2A). On the C-terminal end is the receptor binding domain (RBD). This domain is active extracellularly and is used to bind to host target cells. The RBD contains repetitive oligopeptides (CROPs), that form a hairpin followed by a loop (126-128). Each toxin uses different receptors and may use more than one receptor (129-133). Once bound, the toxins use receptor-mediated endocytosis to enter the host cell (134, 135). After entry, the toxins are contained in endosomal compartments. When the compartments are acidified, the low pH induces structural changes in the toxin that force the central translocation domain, a small hydrophobic stretch beside the RBD, to insert into the endosomal membrane (134-137). The actual mechanism of transport of the CPD and the N-terminal glucosyltransferase domain (GTD) across the endosomal membrane into the cytosol is not well defined. Once the toxin reaches the cytosolic side of the membrane, the cysteine protease domain autoproteolytically processes the toxin (138). Cleavage of the toxin releases the GTD into the cytosol.

The GTD is the domain responsible for the pathology of both TcdA and TcdB. It is active in the cytosol and glycosylates Rho GTPases, such as Rac and Cdc42 (139-141). It uses UDP-glucose as a co-substrate and forms α-anomeric linkages (140, 141). These linkages ensure that the glycosylation causes irreversible damage, as human cells do not have the glucosidases capable of cleaving them in the cytosol (111). Rho GTPases play a role in several cell processes, including regulation of the cytoskeleton, motile processes, and intracellular traffic (142-144). Their inactivation leads to serious consequences in the cell. There is a disruption of the cytoskeleton and tight junctions (113, 145). Disruption of the cytoskeleton and loss of stress fibers, leads to changes in cell morphology. The cell shrinks while maintaining irregular neurite-like extensions (146, 147). This affects cell-cell contact and cell adhesion, leading to a loss of

barrier function in the intestine (147-151). The actin effects of the toxins, as well as TcdAs' ability to activate caspases-3, 8 and 9 induces apoptosis in target cells (152-156).

Both toxins activate the inflammasome, leading to an influx of immune cells, fluid accumulation and tissue destruction (157). TcdB induces pyroptosis through activation of the pyrin inflammasome (158, 159). Pyroptosis is the swelling of the cell until lysis, with a release of cellular contents, which drives strong inflammation. These results are not dependent on the GTD in a high toxin dose ileal loop model (160). The use of toxin doses that may exceed physiological levels during CDI in this model, does call into question how much the inflammasome contributes to CDI pathology. The activation of the inflammasome releases IL-1β and IL-18 into the surrounding tissues. IL-1β secretion induces local responses, such as promoting leukocyte infiltration, IL-6 and IL-8 production, and systemic responses, such as activating lymphocytes and inducing a fever (161). IL-18 promotes T_H1 cell polarization in T cells and induces an increase in IFNγ production. Both toxins can also activate mast cells which contribute to neutrophil recruitment (162, 163).

1.3.1.2 Comparing toxins A and B

There is a long-standing debate about the relative contributions of these toxins to CDI in an attempt to discern which is more potent. Some of the confusion stems from their different potencies in animal models. Some groups have shown that TcdA is more potent in increasing secretion, mucosa damage, and inflammation (164-166). While others have demonstrated that TcdB is more important in innate immune and inflammatory responses (167). Both toxins play an active role in infection, however it appears rodents are more susceptible to TcdA *in vivo*, while TcdB is more potent in cell culture assays (10^2-10^3-fold) such as human colonic epithelial cells (168, 169). Strains secreting only one of TcdA or TcdB have been isolated from humans. $TcdA^-TcdB^+$ strains have been isolated from patients since the 1990s and are considered more common than $TcdA^+TcdB^-$ strains (170-172). However, a recent study in Boston, MA, found 3.7%-7.5% of CDI patients had more TcdA than TcdB in their stool (173). While the strains isolated from these patients had genomic sequences for both toxins, they produced more TcdA *in vitro*. Similar strains were found in asymptomatic carriers in the study. Data from human studies,

including clinical trials, point towards TcdB playing a more dominant role in CDI pathogenesis. These studies are discussed in detail in **Section 1.4.2.1** and **Section 1.6.4**.

C. difficile Toxin A has several receptors on human IECs. The main receptor appears to be the glycan sequence GalNAc-(1,3)-Beta-Gal-(1,4)-Beta-GlcNAc (174). TcdA also uses Lewis I, X and Y glycan sequences as receptors (175, 176). In the colon, a heat shock protein, gp96, may be used as a coreceptor by TcdA (177). After binding to its receptor, TcdA is endocytosed in a PACSIN2/Syndapin-II mediated manner (178). PACSIN2 is a protein that regulates the actin cytoskeleton and is involved in receptor mediated endocytosis through its interactions with dynamin (179, 180).

TcdB has several cell receptors including chondroitin sulfate proteoglycan 4 (CSPG4) (133, 181). CSPG4 is a membrane bound proteoglycan with a single transmembrane domain (182). Wnt Receptor Frizzled (FZD) proteins are able to bind and uptake TcdB, but not through binding at the CROP domains. Once bound, TcdB uptake is strictly dependent on clatherin-mediated endocytosis (183).

TcdB accounts for some of the increased virulence in the NAP1 *C. difficile* isolates compared to historical strains. The TcdB produced by NAP1 strains has broader tropism and cytotoxicity (25). Its' conformational changes that allow insertion into the endosomal membrane occur at a higher pH, allowing more rapid entry into the cytosol, perhaps contributing to more severe illness.

1.3.2 Binary toxin

As noted above, 5-30% of *C. difficile* strains produce binary toxin, or *C. difficile* transferase toxin (CDT) (112). When administered intraperitoneally (IP), CDT can be toxic to mice in high doses, however several groups have shown that $TcdA^{-}TcdB^{-}CDT^{+}$ strains of *C. difficile* are non-pathogenic in rodents (113, 168, 184). CDT triggers the formation of microtubule protrusions on IECs leading to enhanced colonization of the gut by *C. difficile* (185).

CDT has two main components, CDTa and CDTb (Fig 1.2B). CDTb binds the host cells and can be divided into 4 domains (186). From the N-terminal towards the C-terminal, there is activation domain I, the membrane insertion and pore formation domain, domain III, which is responsible for oligomerization and domain IV, the RBD. The RBD is activated by cleavage of domain I, which releases a 20kDa fragment, allowing oligomerization and the formation of heptamers. CDTa is the catalytic component, with two identically folded domains (187). The N-terminal domain interacts with CDTb and the C-terminal domain is the catalytic domain. CDT binds lipolysis-stimulated lipoprotein receptor (LSR), which is involved in lipoprotein clearance and is highly expressed in the liver (188-191). It is also expressed in the intestine, kidneys, and lungs, and is involved in the formation of tight junctions (192). After CDT binds LSR, LSR accumulates in lipid rafts, and CDT oligomerizes (193-195). Once internalised, CDT inserts into the vesical membrane when the compartment pH drops (196). CDTa is an actin-specific ADP-ribosyltransferase, that inhibits actin polymerization and depolymerizes actin filaments (112, 197). This reduces actin-dependent processes such as barrier functions in epithelial cells, migration, phagocytosis, endocytosis and secretion. There is a re-routing of fibronectin and other extracellular matrix proteins from the basal membrane to the apical membrane in IECs, forming the previously mentioned protrusions and increasing bacterial binding to the cell (198). CDT can also inhibit eosinophilic responses in mice (199, 200).

1.4 IMMUNE RESPONSE TO *CLOSTRIDIOIDES DIFFICILE*

There are three lines of defense against *C. difficile*, the epithelial barrier, the innate immune response, and the adaptive immune response. TcdA and TcdB break down the epithelial barrier, through manipulation of the cytoskeleton leading to the loss of tight junctions (113, 145). The innate and adaptive responses elicited by *C. difficile* will be discussed below.

1.4.1 Innate immune response

As the first host cell to come in contact with *C. difficile* and its toxins, IECs play a vital role in the innate response. The effects of Rho-GTPase glycosylation by the toxins has been discussed, however the toxins also mediate glycosylation-independent effects in IECs (159). Both toxins activate inflammatory signaling pathways and secretion of pro-inflammatory cytokines and chemokines. Intracellular toxins trigger the production of reactive oxygen species

(ROS) (201). ROS not only have bactericidal effects, but they activate anti-bacterial defensins, such as human α-defensin 5, which directly inactivates TcdB *in vitro* (202, 203). TcdA-triggered production of ROS activates the nuclear factor-kappa B (NF-κB) pathway (204, 205). This leads to the secretion of IL-8 by IECs (155, 206-208). IL-8 plays a role in neutrophil recruitment to the colonic epithelium and lumen. *In vitro* studies have also shown that TcdA triggers the release of growth-related oncogene α (GRO-α) and monocyte chemo-attractant protein 1 (MCP-1) from IECs (209). GRO-α is a chemoattractant and activator of neutrophils, while MCP-1 acts a chemoattractant for monocytes, memory T cells and Natural Killer (NK) cells (210, 211). A study using human colonic biopsies identified a strong increase in IL-8, IL-1β, and IFNγ production by IECs during CDI (212). Interestingly, NAP1 infection generated a significant increase in IL-8 compared to a historical strain. IL-1β and IFNγ are both Th1 cytokines that typically promote inflammation.

Once the intestinal epithelial barrier has been breached, *C. difficile* toxins can encounter mucosal resident immune cells. Both TcdA and TcdB have been shown to interact with mast cells to increase the release of IL-8 and neutrophil recruitment (162, 163). *In vitro* both toxins activate the inflammasome in human monocytes and mouse macrophages leading to the release of IL-1β (160). A follow up study in apoptosis-associated speck-like protein containing a CARD (ASC) adaptor protein knock out (KO) mice, which are unable to form canonical inflammasomes, found reduced inflammation and damage from toxin injection. TcdB can also activate the pyrin inflammasome, but this is dependent on GT activity (213). TcdA exposure results in the maturation of bone-marrow-derived dendritic cells (BMDCs) *in vitro* (214). Further exposure to *C. difficile* elicits IL-12, IL-10 and IL-1β production from the mature BMDCs (212). These BMDCs induced T cell proliferation and skewed towards Th1 and Th17 responses.

C. difficile also activates innate immunity in a toxin independent fashion. *C. difficile* is a potent stimulator of nucleotide-binding oligomerization domain protein family 1 (NOD1), an intracellular pattern recognition receptor (PRR) that recognizes peptidoglycan. *In vitro* NOD1 activation leads to increased GRO-α and IL-6 production in mouse bone-marrow derived macrophages (215). *In vivo* $NOD1^{-/-}$ mice have similar epithelial damage to WT controls, but

they have increased mortality. This appears to be a result of decreased GRO-α expression, neutrophil recruitment, and bacterial clearance. Another family of PRRs are toll-like receptors (TLRs), which also recognize *C. difficile* pathogen associated molecular patterns (PAMPs), namely TLR4 recognizes SLPs and TLR5 interacts with flagella (216-219). TLR4 interaction with SLPs is dependent on them being full length, having both HMW-SLPs and LMW-SLPs. TLR4 activation by SLPs matures dendritic cells (216). *In vitro* SLP treated human monocyte derived dendritic cells (MDDC) release a mix of pro-inflammatory and anti-inflammatory cytokines (IL-12 and IL-10) inducing a mixed Th1/Th2 response in T cells (217). In the colon, TLR5 on IECs is mostly on the basolateral side (220). *In vitro C. difficile* flagellin induces IL-8 expression in TLR5-expressing Caco-2 cells, this response is enhanced if cells are pre-treated with TcdB (218, 219). Mice infected with *C. difficile* lacking flagellin have reduced gut inflammation.

Neutrophils play a complicated role in CDI (221). They are a significant source of intestinal damage caused during CDI. Severe CDI is characterised by pseudomembrane formation and heavy neutrophil infiltration in the colonic epithelium (222). However, a dysregulated neutrophil response is also harmful. Increased neutrophils in the peripheral blood is linked to severe CDI and poor prognosis (23, 223, 224). On the other hand, humans with neutropenia are at higher risk of developing CDI and mice with neutrophil ablation experience higher pathogen burden and mortality to CDI (215, 224-226). *C. difficile* toxins activate neutrophils through formyl peptide receptor 1 eliciting ROS generation (227). Neutrophils also phagocytose complement or antibody coated *C. difficile*, aiding in bacterial clearance (228). In strong association with ROS formation and activation, neutrophils release neutrophil extracellular traps (NETs) to aid in bacterial clearance (229-232). The presence and the role of NETs in CDI has yet to be elucidated (221). Neutrophils also play a key role in healing after some forms of gut inflammation (229-231). Healing the gut and returning to gut homeostasis are crucial for reducing morbidity and mortality after CDI. However, it has yet to be determined how neutrophils influence gut healing after CDI. (221, 233-235).

1.4.2 Adaptive immune responses

1.4.2.1 Humoral responses

Most of the knowledge regarding the adaptive immune response to *C. difficile* focuses on the humoral responses to infection. Both TcdA and TcdB are immunogenic and elicit both systemic and mucosal antibodies against them. While they share a high percentage of sequence homology, they are considered antigenically distinct, as antibodies that neutralize one toxin are unable to neutralize the other (236).

Systemic antibodies against *C. difficile* toxins have been correlated with better disease outcomes. It is important to consider how systemic IgG enters the colonic epithelium. Neonatal IgG Fc receptor (FcRn) transports IgG across the intestinal epithelial barrier (237-239). Although recent data suggest that this transport may be more important in the context of vaccination and challenge rather than during natural infection (237). Gut leakage after cell damage and tight junction loss mediated by *C. difficile* toxins is the most commonly suggested method for lumen access of systemic antibodies (240-242). Around 60% of healthy adults have detectable levels of serum IgG and IgA against TcdA and TcdB (241). A study in children over 6 months old, observed that a similar percentage have *C. difficile*-specific antibodies in their serum (243). This may be related to the high level of *C. difficile* colonization in infants (49). Since a smaller percentage of healthy adults carry *C. difficile*, it is unclear if their antibody titers are a hold-over from childhood infection or a consequence of subsequent subclinical infections/exposures (222, 241). High TcdA-specific IgG titers have been correlated with mild CDI cases, while severe CDI has been associated with low levels of TcdA-specific IgM, IgG2 and IgG3 titers (244-246). In clinical trials using monoclonal antibodies targeting *C. difficile* toxins, the placebo subjects with higher pre-existing TcdB-specific serum antibodies had lower rates of recurrence but this was not the case for participants with pre-existing TcdA-specific antibodies (247, 248). In one study, it was determined that the neutralizing effects of patient serum on TcdA was restricted to IgA1 antibodies (249). In addition to toxin-specific antibodies, CDI also elicits antibodies against *C. difficile* surface proteins, most prominently the LMW-SLPs (250-252). While antibody titers against SLPs are similar in patients with CDI, asymptomatic carriers and non-carriers, low SLP-specific IgM titers are correlated with recurrent infections (253).

In the colonic mucosa, activated DCs promote a mixed Th1/Th2 response during CDI. The Th2 response elicits a mucosal adaptive response with IgA class switching in B cells. Fecal IgA in humans neutralizes *C. difficile* toxins (254). High TcdA-specific IgA titers in the stool have been associated with lower risk of recurrence (244). Low levels of TcdB-specific IgA antibodies in the stool early in infection, have been associated with susceptibility to CDI (255).

1.4.2.2 Cell mediated immunity

There are relatively little data on cell-mediated responses to CDI (256). However, increased risk of CDI in HIV/AIDS patients with <50 $CD4^+$ T cells/μL in the blood suggests that T cells play a role in protection from CDI (257). In fact, a recent study by Cook et al demonstrated that TcdB-specific T cell responses correlate better with disease severity and recurrence than antibody responses (258). *In vitro* BMDCs exposed to paraformaldehyde fixed historical strains of *C. difficile* elicit a predominantly Th17 response in splenocytes, however exposure to a NAP1 strain elicits a predominantly Th1 response (212, 259). Studies performed in humans examining $CD4^+$ T cells in the blood have yielded conflicting results. One group correlated shifts to Th17 and Th2 dominated responses with severe disease (260). While others observed correlations between higher Th1/Th2 and Th1/Th17 ratios in blood $CD4^+$ T cells and more severe disease (258, 261). These differences could be influenced by the time of sampling during infection, but more research is needed to define how T cells protect or harm the host during CDI.

The response to CDI of two innate-like T cells has also been studied. Mucosal-resident γδ T cells have been shown to play a role in protecting neonatal mice from CDI through IL-17 production (262). Mucosa-associated invariant T (MAIT) cells respond to *C. difficile* by releasing IFNγ, granzyme B and perforin (263). This response is increased when these cells are exposed to NAP1 strains.

1.4.3 Responses to recurrence

The differences in the immune response to recurrent CDI compared to primary infection are not well studied. As mentioned above, several studies have linked decreased antibody titers with higher risk of recurrent CDI. Since most studies compare patients with primary CDI to

patients with recurrent CDI, it is also possible that the differences observed are what predisposed patients to recurrent CDI in the first place. Unfortunately, the few studies performed have yielded conflicting results. Yacyshyn et al observed increased numbers of lymphocytes, and specifically, $Foxp3^+$ and IL-17^+ T cells in the blood of patients with recurrent CDI (264). Cook et al recently observed decreased TcdB-specific Th17 $CD4^+$ T cells in patients with recurrent CDI (258). Yacyshyn et al did not test the antigen specificity of the immune cells, so it is possible that recurrent CDI patients have increased general inflammation, with a decreased TcdB-specific response. At the mucosal level, Johal et al reported a reduction in colonic IgA^+ plasma cells in intestinal biopsies from patients with recurrent CDI (265).

1.4.4 Immunity in asymptomatic carriers

While non-toxigenic *C. difficile* strains exist, and can colonize humans without causing CDI, toxigenic strains have also been found in asymptomatic carriers. The mechanism of the protection in asymptomatic carriers is not yet fully elucidated but, a healthy microbiome clearly plays a role, and prior immunity to *C. difficile* is hypothesized to be beneficial. Data on antibody responses against *C. difficile* in the setting of asymptomatic carriage have been contradictory. Johnson et al observed decreased TcdA-specific IgG and IgA in the serum of asymptomatic carriers compared to patients experiencing CDI (266). This observation has been extended to IgA in the intestinal lumen, as well as serum IgG and IgM against other *C. difficile* antigens (267). However, other groups have observed increased antibody titers against *C. difficile* in asymptomatic carriers compared to symptomatic individuals. Increased TcdA-specific IgG titers and somatic cell antigen-specific IgA and IgM in the serum of asymptomatic carriers compared to patients with active CDI have been observed (268, 269). Unfortunately, the data regarding asymptomatic carriers is conflicting and does not address the role of cell-mediated responses.

1.5 *CLOSTRIDIOIDES DIFFICILE* INFECTION IN THE ELDERLY

The elderly are most at risk both of developing *C. difficile* infection and for experiencing more severe symptoms and/or outcomes from CDI. While the elevated risk may be related to co-morbid conditions, such as higher antibiotic use, some epidemiology studies have shown that advanced age is a risk factor even when the analysis controls for co-morbidities (222, 270). This is particularly true when patients are infected by the NAP1 strain. The increased susceptibility to

CDI has been attributed to two main factors; immunosenescence and changes in the intestinal microbiota.

1.5.1 Immunosenescence

As people age, the number of innate immune cells remains fairly constant, but the function of these cells decreases. While neutrophils play a vital role in the response to CDI, several studies have shown that they have decreased function in older individuals. *In vitro*, neutrophils from older patients have decreased chemotaxis, recruitment, and defective egress from inflamed tissues (271, 272). They also have impaired intracellular killing of pathogens and formation of NETs (273). Phagocytosis of *C. difficile* specifically may require the assistance of complement and neutrophils isolated from elderly people have decreased phagocytosis of *C. difficile* compared to young healthy people (274). However, when serum from the young volunteers is added to aged neutrophils *in vitro*, phagocytosis is rescued. When the serum from younger subjects is heat-inactivated however, the effect is lost suggesting complement is the necessary component (228).

When aged mice are challenged with VPI 10463, they have significantly less colonic pathology than young mice, with no difference in bacterial colonization (275). A smaller number of $CD45^+$ leukocytes infiltrate the colonic lamina propria 2 days post infection (dpi) in aged mice. Aged mice have an increase in peripheral eosinophils during CDI, suggesting that the eosinophils may not be recruited to the gut after activation during active infection. Aged mice also differ from younger animals in their cytokine response to *C. difficile* infection. Old mice have increased levels of IL-17A in the serum and decreased GRO-α, and no increase in IL-5 which is seen in young mice. These *C. difficile*-specific observations are consistent with the large body of literature on immunosenescence describing dysregulated cytokine and chemokine production in older individuals. Inflamm-aging is the term used to describe the persistent low-grade state of inflammation that is often observed in the elderly (276, 277). Elderly patients have increased levels of TNF-α, IL-6 and IL-8 in the serum compared to young adults (278, 279).

As outlined above, aspects of the humoral response to *C. difficile* are associated with protection, and it is well known that humoral responses are decreased during immunosenescence.

The elderly tend to have fewer naïve B cells in the peripheral blood, by both number and percentage (280). As individuals age, there is a contraction of the B cell repertoire and impaired ability to class switch and produce high affinity immunoglobulins (Ig) (281). Serum IgA levels are often elevated, but unfortunately it is monomeric IgA, so it is unable to be transported to the intestinal lumen (282). These effects of aging on humoral responses likely increase the risk and impact of CDI in older individuals. Indeed, low antibodies levels against *C. difficile* in the elderly are associated with greater risk of CDI and the inability to mount an IgM or IgG response to infection is associated with higher risk of recurrence (245).

1.5.2 Intestinal microbiota

Several studies have characterized the elderly gut microbiome as having fewer competing anaerobes, such as Bacteroides, Prevotella and Lactobacillus (283, 284). Admission to and residence in LTCFs are associated with lower gut microbiome diversity (285). These changes open an environmental niche that allows *C. difficile* to colonize and expand in the gut. While the intestinal microbiota of young adults is dominated by the Firmicutes phylum, the elderly microbiota is dominated by the Bacteroidetes phylum (286). The use of some antibiotics can markedly decrease the abundance of the Firmicutes population in the gut, so some of the changes in the microbiome that occur as we age, may mirror the changes driven by antibiotic use. There is also a decrease in abundance of Bifidobacteria in the elderly, which is more pronounced in elderly patients who have had CDI.

1.5.3 Additional factors for increased risk

Altered physiology in the gastrointestinal tract may also play a role in the increased risk for CDI in the elderly. The elderly have decreased stomach acidity, mucus and bicarbonate secretion, and colonic motility (287). They can also have impaired blood flow to the gut mucosa (287). Poor functional status has been identified as a risk factor for CDI and more severe outcomes (14, 288). Functional status is related to mobility, independence in performing activities of daily living (ADL) and cognitive function. All of these factors could contribute to the development of more severe CDI symptoms.

1.5.4 Outcomes

The easiest outcomes to measure after a primary CDI are mortality and recurrence. Overall, 92% of deaths caused by CDI are in patients 65 years or older (289). During the NAP1 epidemic, 30 day and 1-year mortality after CDI were increased in patients 70 years or older (290). As discussed above, immunosenescence contributes to increased risk of recurrence as well. Despite the emphasis on CDI-associated mortality, other outcomes that are more difficult to measure are just as important in the lives of CDI patients. After an acute episode of CDI in an elderly patient, a common adverse outcome is a precipitous decline in functional status (22, 291). Many older patients experience overall debility and loss of independence in ADLs after an episode of CDI. Patients hospitalized with CDI have increased rates of discharge to LTCFs and other primary care facilities (292, 293). These adverse outcomes can have a drastic impact on the quality of life of patients after recovering from CDI.

1.6 *CLOSTRIDIOIDES DIFFICILE* TREATMENTS

While antibiotics are the current first line treatment for CDI, a small number of other treatments are also available. In addition, more experimental treatments for *C. difficile* will be discussed in this section.

1.6.1 Antibiotics

Metronidazole was the antibiotic of choice for CDI in the 1980s (294). Although it is less expensive than the alternatives, several clinical trials over the last 15 years have demonstrated metronidazole to be inferior to vancomycin and fidaxomicin (295-297). It has higher treatment failure rates and more adverse side effects (12). However, after intravenous (IV) injection, metronidazole penetrates the colon in minutes, so its' use is still recommended in cases of fulminant CDI (298).

The current recommended treatments include the use of vancomycin or fidaxomicin. In two Phase III clinical trials, fidaxomicin was found to be non-inferior to vancomycin, with both having cure rates around 90% (299, 300). Fidaxomicin has fewer detrimental effects on the microbiome and a decreased rate of recurrence (301). In one small study, fidaxomicin was more effective at reducing the number of spores in patients' stool (302). Fidaxomicin has a prolonged

post-antibiotic effect, meaning it can be delivered twice daily, compared to vancomycin's four doses daily (294, 303). Despite concerns of vancomycin-resistant enterococci, the high cost of fidaxomicin means that it cannot be recommended over the use of vancomycin in all cases.

1.6.2 Probiotics

It is not yet fully clear if taking probiotics can prevent CDI. This lack of clarity is likely attributable to the variety of probiotics and dosages tested in the published studies. Probiotics can contain a single live culture, or a mix of several cultures, usually *Lactobacillus* strains, *Bifidobacterium* strains or *Saccharomyces boulardii* (304, 305). *Lactobacillus* and *Bifidobacterium* strains inhibit toxin adhesion to IECs *in vitro* (306, 307). In the absence of clear evidence, investigators sometimes interpret results in very different ways, for example, in a placebo-controlled randomized trial, probiotic administration (mixture of *Lactobacillus* and *Bifidobacterium*) did not reduce CDI rates in patients 65 and older (308). However, in a recent systematic review that included this negative trial, Goldenberg et al concluded that "moderate quality evidence suggests that probiotics (*Lactobacillus*, *Saccharomyces*, or a mixture) are both safe and effective for preventing *C. difficile*-associated diarrhea" (309). Another strategy is the administration of a non-toxigenic *C. difficile* strain during CDI which appeared to prevent CDI recurrence in at least one randomised control trial (310, 311). Recently, Vedantam et al engineered two strains of *Lactobacillus* that express a chimeric SlpA from *C. difficile*, allowing them to outcompete *C. difficile* for gut adhesion (312). Three oral doses of the modified *Lactobacilli* prior to infection protected piglets from CDI. Clearly, more detailed studies are needed to clearly identify the possible benefits of probiotics in the context of CDI.

1.6.3 Fecal microbiota transplants

Fecal microbiota transplant (FMT) is a therapy that has been highly publicised. It was an emerging therapy in the early 2010s that aimed to restore the intestinal microbiota through the installation of the microbiota of a healthy donor. However, the procedure has not been standardised and is considered by some to be quite 'invasive'. The donor microbiome can be delivered to the upper or lower ends of the gastrointestinal tract by nasogastric tube, endoscopy, gastroscopy, colonoscopy, enema or rectal tube (313). FMTs are generally recommended for treatment for multiple recurrent CDI (314, 315). Prospective observational studies suggest a

single FMT is 70-80% effective at resolving CDI (294, 316). In randomised controlled trials, a single FMT has been 65-80% effective, with a second FMT in non-responders increasing efficacy to 83-94% (317, 318). A recent study by Cook et al, observed an increased proportion of TcdB-specific Th17 T cells and increased toxin-specific IgG and IgA titers in the blood after FMT treatment (319). There was no change in TCR repertoire and a decreased proportion of TcdB-specific Th2 T cells. To reduce the invasiveness of FMT, oral capsules containing lyophilized bacteria are being developed. In a very small study of 20 patients, one capsule resolved CDI in 70% of patients, and after a second capsule 90% of patients were cured (320).

1.6.4 Antibodies

Polyclonal antibodies against *C. difficile* were first tested in animals in 1982 (236). Rabbit antiserum raised against one toxin effectively protected mice from that toxin but had no cross-protection. Polyclonal antibodies were first used as passive immunotherapy against CDI in people in 1991 (321). TcdA neutralizing IgG delivered IV was associated with resolution of CDI in both children and adults. Recently, in a very small study, polyvalent Ig had a therapeutic effect in 41% of patients (322). The main concern with the use of polyclonal antibodies is the lack of standardization from lot to lot. Different preparations can have varying levels of IgG subclasses and well as differences in neutralization ability (322).

Monoclonal antibody preparations do not have this issue. Two humanized monoclonal antibodies targeting the RBDs of TcdA (actoxumab) and TcdB (bezlotoxumab) have undergone clinical trials (58). In a Phase I clinical trial, actoxumab had no side effects after IV injection in healthy adults and did not elicit a response against human IgG (323). The half-life of actoxumab is 25-31 days. In Phase II/III clinical trials, the antibodies were delivered IV alone or in combination with each other, and the primary outcome measured was recurrence (324, 325). Bezlotoxumab, was most effective at reducing recurrence rates when delivered without actoxumab and has since been approved by the FDA for use in humans (326). It is delivered in one IV dose in conjunction with standard antibiotic treatment to prevent recurrent CDI. It is most beneficial for patients who are at high risk for recurrence and is not immunogenic (327, 328).

1.6.5 Surgical interventions

Around 3-10% of CDI patients will progress to fulminant CDI (7). Fulminant CDI includes the most severe complications of CDI, such as pseudomembrane colitis, toxic megacolon, intestinal perforation, and sepsis. These patients may require emergency surgical intervention for treatment. In a retrospective observational cohort study, colectomy was of greatest benefit in patients older than 65 with high leukocytosis (329). However, the mortality rates following colectomy are still very high, ranging from 35-80% (7, 330). A loop ileostomy provides an alternative surgical intervention, that is colon-saving. In one small study of fulminant CDI patients, mortality was reduced to 19% in those treated with a loop ileostomy compared to 50% mortality in those who received a colectomy (331). In a larger, retrospective multicenter study, patients treated with a loop ileostomy had increased survival compared to patients who received a colectomy (82.8% vs 60.3%) (332).

1.6.6 Antigermination strategies

A novel prevention strategy that is currently being investigated is the use of synthetic bile acid analogs to prevent *C. difficile* spore germination in the gastrointestinal tract. As discussed in **Section 1.1.6**, bile acids have the potential to both stimulate and block germination. Synthetic bile acid analogs that mimic inhibitory bile acids have been tested in rodent models. In mice, they reduce CDI severity at low doses (333). In hamsters, when given in combination with vancomycin, synthetic bile acid analogs prevent CDI (334). They also have a much smaller detrimental effect on the microbiota compared to antibiotics, suggesting they may reduce the risk of recurrence. These novel drug candidates are still under development, and their safety profile is unclear, as some bile acids have been implicated in the development of colon cancer (76).

1.7 *CLOSTRIDIOIDES DIFFICILE* INFECTION PREVENTION STRATEGIES

As discussed above, the treatment options for *C. difficile* are either not very effective (high recurrence rate), have a high cost, or are highly invasive. The best way to reduce *C. difficile* morbidity and mortality is to prevent infection in the first place. To date, a number of strategies have been used to limit CDI rates in high-risk populations (22). With antibiotic use being the main modifiable risk factor for CDI, antibiotic stewardship is a vital approach for CDI prevention (335-337). There are two basic approaches to antibiotic stewardship: i) reduction of

unnecessary antibiotic use and ii) avoidance of high CDI risk antibiotics, such as clindamycin, cephalosporins and fluoroquinolones. Overall, 50% of antibiotics prescribed in hospitals are thought to be unnecessary or inappropriate and that rate increases to 75% in LTCFs (338, 339). Although the implemention of antibiotic stewardship programs in LTCFs can be challenging due to lack of staff, such programs are effective at reducing CDI cases by 32-52% (335, 336). The use of lower CDI risk antibiotics is associated with up to 60% reduction in CDI cases (340, 341). These strategies work best when implemented with transmission prevention strategies (335). The primary route of transmission for *C. difficile* in health care facilities is on the hands of health care workers (33). The use of proper personal protective equipment, disposable medical equipment, private rooms and cleaning of rooms of CDI patients with sporicidal agents can all help to reduce transmission in health care settings (294). While it is unclear how much transmission occurs from asymptomatic carriers, active screening for CDI upon hospital admittance can reduce CDI cases by as much as 60% (53). While this is not standard practice, such testing may be helpful to implement in outbreak or high CDI burden settings.

1.8 VACCINES TARGETING *CLOSTRIDIOIDES DIFFICILE*

Another strategy for CDI prevention is vaccination. With the high rate of recurrence after treatment and an increasingly large population at risk for CDI, there is a strong need for a vaccine (9, 13). Unfortunately, there are currently no vaccines against *C. difficile* on the market. Three vaccine candidates have undergone Phase II/III clinical trials. These will be discussed in some detail below, as well as several other vaccine candidates currently in pre-clinical testing.

1.8.1 Clinical trial results

The first *C. difficile* vaccine to reach a Phase III clinical trial was Sanofi's *Cdiffense*™. The vaccine is comprised of formalin inactivated TcdA and TcdB with aluminum containing adjuvant delivered intramuscularly (IM). In Phase I clinical trials, the vaccine was administered in 3 doses, 4 weeks apart. The vaccine was well tolerated and immunogenic in young adults and the elderly (342). Seroconversion for TcdB was lower than for TcdA in the elderly (343). For the Phase III clinical trial, 9302 high risk participants were recruited (NTC01887912). All the participants were over the age of 50; and had either had two hospital stays of over 24 h with systemic antibiotic use in the last 12 months or were anticipating a hospital stay of over 72 h for

elective surgery in the next 60 days. By ELISA, 17% and 64% of participants were seropositive for TcdA and TcdB, respectively, prior to vaccination (344). Participants were divided 2:1 in the vaccine and placebo groups, with 6201 individuals receiving the vaccine. The vaccine was delivered in three 100 μg doses, on day 0, 7 and 30. The primary outcome for the study was CDI in the 3 years following vaccination. Although the study was complicated by a 'clinical hold' order for ~10 months due to serious adverse events, it was subsequently determined that they were unrelated to the vaccine, and the study continued. Antigen-specific antibody titers peaked 60 days after vaccination. The titer of TcdA neutralizing antibodies spiked 60 days after vaccination, but TcdB neutralizing titers did not spike. At the first interim analysis when 50 cases of CDI had been identified, 34 cases had occurred in the vaccinated group, and 16 cases had occurred in the placebo control. With a vaccine efficacy of -5.2% [95% CI, -104.1 to 43.5], the study was terminated and further development of this candidate was abandoned.

The only other *C. difficile* vaccine to be tested in a Phase III clinical trial, is Pfizer's PF-06425090. This vaccine uses a non-toxigenic strain of *C. difficile*, that has a mutated *spo0A* gene, to express full length TcdA and TcdB toxoids. The toxoids are genetically modified to prevent GT and autocatalytic activity (345). In Phase I clinical trials, the vaccine was adjuvanted with an aluminum hydroxide-containing formulation and administered IM in three doses, on day 0, month 1 and month 6 (346). The vaccine was both immunogenic and well tolerated in older adults (50-85 years old). For the Phase III clinical trial, ~17500 high risk adults were enrolled (NTC03090191). All participants were over the age of 50 and had increased risk of future contact with healthcare systems or had received systemic antibiotics in the previous 12 weeks. Patients who had previously experienced CDI were excluded from the study and participants were randomized 1:1 into the vaccine and placebo groups. This clinical trial was completed in December 2021, with 42 cases of primary CDI occurring over a 4 year period. While the vaccine was 100% effective at preventing medically attended CDI, a secondary outcome, it failed to meet the primary endpoint of the study, reducing primary CDI after 2 or 3 doses (347). Vaccine efficacy for primary CDI cases decreased from 49% 12 months after the third dose, to 31% by the end of the study. However, the median and average duration of CDI symptoms were reduced 75% and 80% respectively in the vaccinated participants. At the current time, it is unclear

whether or not Pfizer will continue the development of this candidate given these mixed and generally disappointing results.

Finally, Valneva's VAL84 has been tested in a Phase II clinical trial. This vaccine candidate differs slightly from the previously discussed candidates, as it is a fusion protein with the RBD of both TcdA and TcdB (348). In preclinical studies, when delivered IM with aluminum hydroxide (alum), this candidate elicited IgG antibodies against both toxins in mice, hamsters, and monkeys. It protected mice from toxin challenge and hamsters from spore challenge. In a Phase I clinical trial the vaccine candidate was administered IM, with and without alum. Adults received three doses on days 0, 7, and 21 and elderly adults received four doses delivered days 0, 7, 28, and 56 (349). High TcdA- and TcdB-specific IgG antibody titers were elicited in both age groups. The titers in adults peaked at day 28, while they only peaked on day 84 in the elderly. Toxin neutralizing titers correlated well with the IgG titers determined by ELISA. The vaccine was well tolerated at all doses in all participants. Although a Phase II clinical trial (NTC02316470) with adults aged 50-64 and 65+ was successfully completed in October 2015, Valneva has been unable to find a partner to advance the vaccine to a Phase III clinical trial (350-352).

1.8.2 Preclinical vaccines

There are also many *C. difficile* vaccine candidates in various stages of preclinical development. For space and interest, this section will focus on vaccine candidates that target surface proteins of *C. difficile* and/or are delivered to a mucosal surface.

In toxin mediated diseases, there have been several notable successes in targeting the toxins with vaccines, including tetanus, diphtheria and whooping cough (353, 354). As described above, this is the primary strategy that has been used to date in *C. difficile* vaccine development. However, some academic groups have chosen to target cell surface proteins. For example, Bruxelle et al are developing a recombinant SlpA that is delivered intrarectally (IR) with cholera toxin as an adjuvant (355). This strategy elicits serum IgG and fecal IgA. It decreases *C. difficile* colonization in mice 10 days after a sublethal challenge. In a lethal hamster model, the vaccine has a slight protective effect, with 50% of the control group succumbing to infection 2.5 dpi and

50% of vaccinated hamsters succumbing to infection 5 dpi. Overall survival from challenge is similar in both groups (~20%). There has been better success in targeting FliC. Ghose et al are developing an intraperitoneally (IP) delivered alum-adjuvanted recombinant *C. difficile* FliC (356). Three doses protects both mice and hamsters from lethal challenge. Bruxelle et al have also delivered *C. difficile* FliC encapsulated in pectin beads PO to hamsters (357). While no IgG is elicited, there is significant protection of hamsters from lethal challenge. Together these data demonstrate that FliC is a feasible target for vaccination. Some groups hypothesize that targeting surface proteins on *C. difficile* will reduce colonization better than targeting the secreted toxins. Mice against the c-terminal domain of BclA3, a spore surface protein, intranasally (IN) are protected from sublethal challenge (358). Spore burden in the feces is significantly decreased in mice 1 dpi. However, the difference in burden is lost by 2 dpi. This may be attributable to the ability of mice to more easily clear *C. difficile* than both humans and hamsters. Vaccine candidates target reduced colonization to decrease transmission to other susceptible hosts.

As discussed above, IM delivered vaccine candidates eliciting a systemic immune response against *C. difficile* have been the only candidates to undergo testing in late-stage clinical trials. However, many preclinical vaccine candidates target a mucosal response. IN, IR and PO administration routes have been tested, with IN being the most successful without the use of adjuvants or vectors (58). Several groups are working to develop vaccine candidates using bacterial vaccine vectors. For example, one group has generated *Bacillus subtilis* spores that express the RBD of TcdA (359, 360). After PO delivery, the vaccine elicits neutralizing IgA in the feces and IgG in the serum of mice (359). IgA from vaccinated mice prevents *C. difficile* from adhering to human colonic epithelial cells *in vitro* (360). The vaccine candidate reduces *C. difficile* colonization and protects hamsters from lethal challenge. A second group is developing *Lactococcus lactis* strains that express the RBD of TcdA or TcdB (361). When these vaccine candidates are delivered PO and adjuvanted with EMULSIGEN®-D, they do not elicit high IgG titers, but mucosal IgA antibodies are induced. The strain targeting TcdA provides significant protection in mice, while the strain targeting TcdB only provides moderate protection. These studies strongly support the possibility of a successful PO delivered, bacterial-vectored vaccine candidate against *C. difficile*.

1.9 ORAL VACCINATION

There are several orally delivered vaccines on the market, all of which are live attenuated vaccines (362). Oral vaccination has the potential to elicit both systemic and mucosal immune responses depending upon the characteristics of the attenuated organism used (362-364). Such responses are likely to be particularly beneficial for targeting a mucosal pathogen. Overall, 60% of all drugs are delivered orally, as it is the easiest and most patient-accepted route of administration (365, 366). Needle-free delivery eases self-administration, distribution and improves patient compliance (367-369).The main challenges in developing a successful oral vaccine are attributable to the physiology and immunology of the gut. Initially, the antigen needs to be delivered to the intestines intact, after passing through the highly acidic environment of the stomach and the intensely basic environment of the proximal duodenum (365). Then, the antigen needs to be delivered across the mucosal barrier, activate APCs and elicit an immune response without inducing tolerance. The human gut is constantly exposed to a truly vast array of antigens from an early age, so the 'default' immune response to almost all antigens has to be the induction of tolerance to ensure survival. A successful vaccine must overcome this default response to elicit a protective immune response (370, 371). A living but attenuated pathogen used as a vaccine or vaccine vector has the potential to overcome these challenges. Some such organisms are pre-adapted to survive the transit through the upper gastrointestinal tract. Furthermore, they mimic natural infection and contain one or more pathogen associated molecular patterns (PAMPs) that are recognized by the host as 'danger signals'. Older live-attenuated vaccines have caused concerns over strong inflammation, uncontrolled replication, prolonged shedding, and the possibility of reverting to a pathogenic form. However, advances in molecular genetics have allowed us to perform targeted attenuation, leading to the design of safer and more stable live-attenuated vaccine vectors or vaccine candidates.

1.10 *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM AS A VACCINE VECTOR

Excerpt adapted from: Current applications of *Salmonella enterica* serovar Typhimurium as a vaccine vector. Prepared for submission to Frontiers Immunology

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Salmonella species have been studied as candidates for orally delivered live attenuated vaccines or vaccine vectors for decades (372). Although multiple attempts have been made to use Ty21a as a vector to deliver a wide range of foreign antigens, none has been particularly successful to date. Although the repurposing of Ty21a has many attractive features, its relatively modest success as a vaccine raises concerns about its possible utility as an effective vector. While *S.* Typhi causes systemic disease, *Salmonella enterica* serovar Typhimurium is generally restricted to the intestinal mucosa in humans. This poses several benefits when designing a heterologous antigen-expressing vaccine vector. Our recent work has focused on the repurposing of another highly attenuated Salmonella strain as a candidate vaccine vector, specifically *S. enterica* Typhimurium (VPN00009 or YS1646) that was originally developed as a cancer therapeutic. This review will summarize the work done by our lab and by others to explore the possible use of attenuated non-typhoidal *Salmonella* strains as vaccine vectors.

1.10.1 *Salmonella* Biology

Salmonella spp. are Gram-negative bacteria and intracellular pathogens. Though closely related to *Escherichia coli*, all species of *Salmonella* are considered pathogenic (373). The genus *Salmonella* contains two species, *S. bongori* and *S. enterica*, where the former comprises 22 serovars and the latter includes over 2,500 serovars (374). Gastrointestinal and extraintestinal pathovars of vertebrate hosts make up subspecies of *S. enterica*, including *S.* Typhi and *S.* Typhimurium. *Salmonella* Typhi is an oral pathogen that crosses the intestinal mucosa and is disseminated systemically (375). It generally does not trigger a mucosal immune response. *S.* Typhimurium has a very different pathology in humans, however it is used in mice as a model for *S.* Typhi infection. *S.* Typhimurium travels through the stomach and into the small intestine and cecum, where it begins to infect the host. The majority of the bacterial population typically resides in the intestinal lumen; however, a small number of bacteria will infect intestinal epithelial cells (IEC) (376-378). The *Salmonella* pathogenicity island (SPI)-1 Type 3 secretion system (T3SS) is activated by the high osmolarity of the enterocyte villus allowing the bacterial cell to begin secreting effector proteins (379). *S.* Typhimurium specifically targets M cells in

Peyer's patches, and alters tight junctions to increase transepithelial migration (380). SPI-1 T3SS effector proteins force the host cell to macropinocytose *S.* Typhimurium. Intracellular *S.* Typhimurium resides in a *Salmonella*-containing vacuole (SCV) that is maintained by the SPI-2 T3SS. Some *S.* Typhimurium strains are able to escape the SCV and undergo hyper-replication in the cytosol (381).

1.10.2 Attenuating *Salmonella* Typhimurium

There are several methods used for attenuating *Salmonella*. Many of these methods have been thoroughly examined in 2016 and 2018 reviews by Galen et al and Curtiss et al (379, 382). The former review focuses on attenuated strains that have been evaluated in clinical trials, while the latter discusses in more detail the strategies and methods behind each attenuation. The traditional method for attenuation is chemical mutagenesis, forcing strains to develop random mutations. Through examination of strains that were sufficiently attenuated and their mutations, some target genes for attenuation have been identified. RpoS, which is involved in acid tolerance responses, is mutated in the *S.* Typhi Ty21a vaccine (383). Other targets for attenuation include genes involved in resistance to bile, resistance to GI defensins, or that encode cell wall components (382). Attenuation strategies range in their combinations (ie: multiple attenuating mutations in a single strain) and their relative sophistication. Curtiss et al discuss some of the more elegant strategies in their review including delayed expression of attenuation phenotypes. This approach allows the vaccine vector to have an almost wild-type phenotype initially upon vaccination, which becomes more attenuated over time, allowing the host to more easily clear the vaccine (384-388). Among the strategies that can accomplish this goal are strains dependent on auxotrophic supplementation to synthesize LPS O-antigen or outer core or that undergo delayed lysis *in vivo* (389). Another approach is the use of delayed production of an additional foreign antigen that is harmful to the vector itself (390-392).

Sufficient attenuation of *Salmonella* vaccine vectors is vital, however too much attenuation is detrimental to the immune response mounted by the vaccine. *Salmonella* strains still need to maintain the ability to survive until reaching the intestine, cross the mucosal barrier, and replicate in host cells. For oral delivery, survival and some replication in the gut mucosa is necessary to trigger protective responses. For this reason, many groups use attenuated,

hyperinvasive *Salmonella* strains as vaccine vectors. Our lab uses the strain *S.* Typhimurium YS1646, originally derived from YS72. YS72 is hyperinvasive, and has *purI* $^{-}$ and *xyl* $^{-}$ mutations. The mutations attenuate the strain by introducing an auxotrophy for adenine and a loss of capacity to use D-xylose as an energy source, respectively (393). In addition, YS1646 is also *msbB* $^{-}$, reducing its septic shock potential by preventing the addition of a terminal myristyl group to the lipid A domain of lipopolysaccharide (LPS). In a clinical trial for its use as a cancer therapeutic, 22/25 patients cleared the bacteria from their bloodstream 12h after IV injection (394). The bacteria were not shed in the urine or stool of patients. However, inoculation with YS1646 generated a significant increase of IL-1β, TNFα, IL-6 and IL-12 levels in the blood, compared to baseline controls. The bacteria did not succeed as a cancer therapeutic as it was unable to reach tumours and replicate within them. The increase in pro-inflammatory cytokines after injection, suggests that unlike Ty21a, YS1646 will elicit a sufficiently inflammatory response after vaccination (379). This response could be elicited against a heterologous antigen, allowing YS1646 to be used as a vaccine vector. At the time of selection of a vaccine vector for our laboratory, YS1646 did not have any freedom to operate barriers, unlike several other attenuated strains of *S.* Typhimurium. For these reasons, in combination with the documented safety profile and hyperinvasiveness of this strain, we have repurposed it as an orally delivered vaccine vector to target mucosal pathogens such as *Clostridioides difficile*, *Schistosomiasis mansonii*, and *Cryptosporidium parvum*.

1.10.3 Immune Responses to Wild Type *Salmonella* Typhimurium infection

To appreciate the potential of *Salmonella* Typhimurium as a vaccine vector we need to understand the immune responses generated by a wild-type infection, as the responses may be quite similar to the responses generated by a live-attenuated vaccine vector. In the next several sections we will discuss the innate, adaptive, and memory responses to *S.* Typhimurium wild-type infection. This provides a context within which we can examine and predict the responses generated by vaccine vectors. It is important to keep in mind that while *S.* Typhimurium is restricted to the gastrointestinal tract in humans, in mice it spreads systemically and immune cells respond to infection differently depending on the tissue that they reside in.

1.10.3.1 Early Responses

One of the advantages shared by all *Salmonella* vectored vaccines is that they are "auto-adjuvanted" to some extent. As a pathogen, *S.* Typhimurium contains a multitude of pathogen-associated molecular pattern molecules (PAMPs) the host can identify through both surface and intracellular pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are PRRs that encounter invading *Salmonella* early in the infection (395). Upon reaching the lamina propria, *Salmonella* is exposed to the TLRs on the basal membranes of intestinal epithelial cells (IECs) (Fig 1.3). Several of the S. Typhimurium surface components activate TLRs, including lipoproteins (TLR1/2/6), LPS (TLR4), flagellin (TLR5) and proteins present in *Salmonella* biofilm have been shown to activate TLR2 (396, 397). CpG rich elements present in *Salmonella* DNA can also activate TLR9.

Activation of the TLRs leads to cytokine secretion in the host cell, generating a pro-inflammatory environment in the gut mucosa. TLR4 signaling leads to TNFα and IL-6 secretion by the IECs (Fig 1.3) (398). In humans, flagellin activates TLR5 signaling, leading to IL-8 and IL-18 secretion (399). IL-18 secretion is dependent on flagellin recognition (400). IL-18 is a strong stimulator of IFNγ production in mucosal-resident T cells (401). IL-23 is released upon TLR activation, although there are several potential sources for IL-23, the source during *Salmonella* infection is not fully known (396). Macrophages and dendritic cells (DC) produce IL-23 *in vitro*, however T cells, Natural Killer (NK) cells and innate lymphoid cells (ILC) are also potential producers. IL-23 is necessary for IL-17 and IL-22 production by mucosal-resident T cells (402, 403). TLR4 signaling has been shown to increase CD4+ T cell responses to *Salmonella* while TLR5 signaling leads to an increased antibody response to *Salmonella* (404). TNFα, IL-6, and IL-8 all contribute to the recruitment of neutrophils and other inflammatory cells to the site of infection.

As *Salmonella* crosses the gastrointestinal epithelial barrier and generates a strongly inflammatory environment, the bacterial cells are phagocytosed by macrophages and dendritic cells present in the lamina propria (396). Lamina propria macrophages are indispensable for clearing *S.* Typhimurium infections (Fig 1.4) (405). Reactive oxygen species (ROS) produced in infected phagocytes can normally control or reduce bacterial growth (406, 407). However,

actively infected macrophages, in the intestine and other organs, can be polarized to an M2 phenotype by the intracellular *Salmonella*. *S.* Typhimurium in the SCV secretes SteE and subsequently activates STAT3, leading to an M2 phenotype in infected macrophages (408). M2 macrophages are permissive to infection by *Salmonella*, and produce IL-10, IL-4Rα, and have anti-inflammatory properties. Uninfected and infected phagocytes are able to present antigen to naive T cells that are also present in PPs. Uninfected but activated macrophages are able to produce IL-12 and IL-18, stimulating IFNγ-dependent Th1 responses (409). IL-1, IL-6 and IL-23 production induces Th17 responses and recruits and activates neutrophils to the site of inflammation (403).

Upon their arrival in the gastrointestinal tract, neutrophils play a large role in killing the bacterial cells in the lumen (158). Their recruitment is crucial in preventing bacterial dissemination (410-413). However, they can also contribute to immunopathogenesis by causing severe damage to surrounding IECs.

These early responses set the tone for the adaptive responses to a wild-type infection. A Th17/Th1 skewed response in the intestine is generated through cytokine secretion by IECs and macrophages, as well as innate lymphoid cells (ILCs). During infection, ILCs in the gut are also activated. RORγT$^+$ Tbet$^+$ ILCs are activated in the colon, and migrate to the mesenteric lymph nodes, to increase the production of IFNγ in the lymph node (414).

1.10.3.2 Adaptive Responses

CD4$^+$ T cells play a crucial role in the clearance of *Salmonella* Typhimurium infections (415). It is important to note that the T cell responses to *Salmonella* infection in mice differ based on the tissue, although this may also be impacted by the various lifestyles of *Salmonella* in different tissues (416). The number of CD4$^+$ T cells increases in the lamina propria (LP) of mice from 3-7 days post infection (dpi) (Fig 1.5) (405). Through Tim-3 and galectin-9 binding on macrophages, CD4$^+$ T cells are able to activate infected macrophages, increasing CD80 and CD86 expression as well as IL-1β production (405). In turn, macrophages are able to activate CD4$^+$ T cells, increasing CD44 expression and IFNγ secretion. Initially, CD4$^+$ T cells in the colonic LP are Th17 skewed, but by 11 dpi they will become Th1 skewed, expressing T-bet and

secreting IFNγ (417). This transition is not dependent on *Salmonella* persistence but does require the presence of Treg cells in the colonic LP. IFNγ production in the LP and mLN assists bacterial clearance in macrophages by activating ROS production (416). *Salmonella* specific $CD4^+$ T cells can be found in mice in the LP up to 90 days after infection (417). This is something that can be taken advantage of in the use of a *Salmonella*-vectored vaccine targeting a gastrointestinal mucosal pathogen. In the spleen, myeloid-derived suppressor cells ($CD11b^+$ $Gr1^+$) are increased during *S.* Typhimurium infection (418). This cell type harbours the bacteria and induces decreased IL-2 production and increased IFNγ and IL-17 by T cells by 5 days post infection via the iNOS-IFNγ pathway (418).

A recent study examined the response to attenuated *S.* Typhimurium vaccination and subsequent challenge with a wild-type *S.* Typhimurium in pigs (419). As the porcine model has some similarities to infection in humans, it is certainly worth taking note of their findings. The study examined TNFα, IL-17a, and IFNγ production by T cells. The highest frequencies of *Salmonella* specific cytokine secreting $CD4^+$ T cells were found in the jejunum and ileum lamina propria lymphocytes (LPL), after vaccination and challenge. The most significant differences between vaccinated and unvaccinated animals after challenge were in the proportion of multifunctional $CD4^+$ T cells, T cells that are producing more than one of the measured cytokines. A majority of the *Salmonella* specific $CD4^+$ T cells that were expressing cytokines in vaccinated pigs were effector memory cells.

Characterization of B cell responses to *S.* Typhimurium has been limited. Many groups have shown that anti-*Salmonella* antibodies are produced upon infection and are correlated with protection from further disease in mice, pigs and humans (420-423). However, recent discoveries have helped us better understand how these antibodies are produced. There is a strong B cell response to *S.* Typhimurium, but it occurs at extrafollicular sites (EF) in the spleen and there is little to no germinal centre (GC) formation. While Th1 responses are necessary for clearance of *Salmonella*, IL-12 production blocks Tfh differentiation, which in turn leads to a lack of GC formation (424). IgM begins to be produced in the spleen 4 days post infection and peak titers are observed 2 weeks after infection (Fig 1.6a). While IgG responses are delayed, they can be 10x more expansive, and they are predominantly subtype IgG2c in C57BL/6 mice (425). At early

time points, the response appears to be non-specific as only 2% of the B cells are producing detectable specific antibodies against *S.* Typhimurium. However, it is dependent on a large BCR repertoire suggesting that the response is specific, but at early time points the affinity is too low to allow detection of specific antibodies. The response occurs in the absence of TLR2, TLR4, MyD88 and T cell signaling. In contrast to general B cell knowledge, Di Niro et al were able to demonstrate somatic hypermutation occurs in cells in the EF as well as in "GC-like" formations (425).

B cells can also function as competent antigen presenting cells (APCs) under appropriate conditions (Fig 1.6b) (426). During primary *S.* Typhimurium infection, all B cell types are actively infected in the spleen of mice both at early time points of infection and during chronic infection (427). These infected cells are able to cross-process and present antigens, including *Salmonella* antigens as well as heterologous antigens produced by bacterial vectors. Antigen processing and presentation requires both the cytosolic and vacuolar pathways. Actively infected B cells upregulate the co-stimulatory molecules CD40, CD80 and CD86, as well as the inhibitory molecule PD-L1. This finding has led some groups to suggest that PD-L1 expression on B cells allows *Salmonella* to establish B cells as a niche for chronic infection in mice.

The adaptive responses to *S.* Typhimurium infections are important in pathogen clearance and the development of a memory response. $CD4^+$ T cells in the intestine are initially Th17 skewed, but eventually become Th1 skewed. The Th1 skewing allows $CD4^+$ T cells to activate ROS production in macrophages, allowing for intracellular bacterial clearance. In mice, infected B cells in the spleen act as APCs, however this may not be the case in human infections, as *S.* Typhimurium generally is constrained to the intestine in humans. *S.* Typhimurium infection generates IgG responses, although the development of plasma cells is unconventional. The most important immune responses generated by a vaccine vector are the memory responses. When developing vaccines, they need to generate long-lasting and effective responses against the pathogen.

1.10.3.3 Memory Responses

Our knowledge on the long-term memory responses generated by attenuated *Salmonella* Typhimurium strains is limited. A recent study examined the long-term responses generated by vaccination by the *S.* Typhi strain Ty21a in humans. 1.5 years after vaccination anti-LPS IgG in the blood was twice as high as unvaccinated controls (428). There were increased levels of FliC-responsive $CD4^+$ and $CD8^+$ T cells in the blood of vaccinated patients. There was also an increase in polyfunctionality in the responding $CD4^+$ T cells in the blood. However, they found no differences in anti-LPS IgA titers in the serum or in antigen-specific $CD4^+$ and $CD8^+$ T cell numbers at the duodenal mucosa between unvaccinated and vaccinated patients.

Combined with knowledge from studies with a *S.* Typhimurium based vaccine technology in mice, we can gain some ideas of what to expect from an attenuated *S.* Typhimurium vaccine vector. Others have published studies using generalized modules of membrane antigen (GMMA) from *S.* Typhimurium. GMMAs are outer membrane vesicles (OMVs) released by genetically modified bacteria. They include LPS, porins and other antigens found on the outer membrane of the bacterial cell. With one dose of GMMAs, researchers found strong B cell responses in both the spleen and bone marrow of mice 203 days (29 weeks) after vaccination (429). When delivered twice 10 weeks apart, with alhydrogel as an adjuvant, GMMAs were able to generate anti-O antigen IgG in the serum and intestines of mice for up to 28 weeks after the 2nd dose (430). There were no significant differences in IgA production compared to control mice at 28 weeks after the 2nd dose.

We can hypothesize that *S.* Typhimurium has the capacity to induce long term responses with IgG^+ B cells, $CD4^+$ and $CD8^+$ T cells. A study in young Malawian children examining the immune responses to natural *S.* Typhimurium infection showed that the frequency of $CD4^+$ T cells in the blood responding to *S.* Typhimurium peaks at 13 months and then begins to decrease in frequency (420). Although the young age of the participants in this study should be considered, it does suggest that we cannot depend on studies of the long-term response to Ty21a to provide complete answers for an attenuated *S.* Typhimurium vaccine vector. This does leave us with a rather unsatisfying answer to the most important question for choosing to use *S.* Typhimurium as a vaccine vector. Hopefully research groups continue to address the memory

responses generated by attenuated *S.* Typhimurium. In our lab, 3 oral doses of YS1646 targeting *Clostridioides difficile* delivered in one week, protected mice from infection 6 months after vaccination. The immune responses generated by this vaccine will be discussed in more details below.

1.10.4 Considerations of *Salmonella* as a vaccine vector

Perhaps the most important consideration when choosing *Salmonella* Typhimurium as a vaccine vector is the type of immune responses generated by the vector. Throughout this paper we have highlighted that responses to *S.* Typhimurium, both a wild-type pathogen and an attenuated vaccine vector, are predominantly Th1 biased, with some early Th17 responses. Anatomically, most responses are generated in the gastrointestinal tract mucosa, with some systemic responses. When choosing pathogens to target with this vaccine vector, this needs to be at the forefront. If Th2 responses are necessary for pathogen control or clearance, multimodal vaccination and/or the addition of Th2 skewing adjuvants may be necessary for protection.

As we move forward with the development of *Salmonella* vectored vaccines, we need to keep a few safety concerns in mind such as whether a strain is safe to be administered to immunocompromised or elderly patients. As mentioned earlier in this review, neutrophils play a large role and $CD4^+$ T cells are essential for clearance of wild type *Salmonella* Typhimurium infection. Patients with low $CD4^+$ T cell counts may struggle to clear a live-attenuated vaccine vector, perhaps leading to adverse events (ie. systemic infection) or prolonged bacterial shedding. Immunosenescence decreases neutrophil function (222, 271-273). This could affect the response generated by the vaccine against the heterologous antigen and could also put elderly patients at risk for more adverse events after vaccination. These concerns are especially important for the development of vaccines targeting immunocompromised individuals, the elderly or populations with high rates of immunocompromising diseases, such as HIV. Bacterial shedding is a risk when developing a live attenuated vaccine. As wild-type *Salmonella* Typhimurium can cause chronic illness, it's also important to ensure that vaccine vectors are appropriately attenuated to allow complete clearance of the vaccine. Ty21a vaccination can lead to some transient bacterial shedding, with almost all shedding being resolved by day 4 after vaccination (431). Both of these issues will need to be addressed through clinical trials. A

backup safety feature in the vaccines for these clinical trials is that they should be antibiotic susceptible. Allowing the trial to clear the vaccine vector from patients if they are unable to clear it on their own.

Over the last decade, we have advanced in leaps and bounds in molecular genetics. This has provided new tools for editing live attenuated vectors. We are no longer dependent on using plasmid-based systems for production of heterologous antigen. This is particularly important for several reasons. While groups have developed methods for maintaining plasmids, including the development of balanced lethal vector-host systems, a plasmid is still a mobile element. Lethal vector-host systems use the deletion of a gene required for survival, making an auxotrophic bacteria (382). The survival gene can then be reintroduced on the plasmid with the heterologous antigen of interest. This is an elegant solution to the issue of the complete inappropriateness that would be giving people a live attenuated vector with an antibiotic resistance gene on a mobile element. However, plasmids can also have varying copy numbers per bacterial cell and can also be lost in the absence of selective pressure. This inconsistency could be problematic during large-scale production of vectored vaccines. Alternatively to plasmids, genes expressing heterologous antigen can now be chromosomally integrated through several methods. Chromosomal integration provides a stable, antibiotic susceptible bacterial vector. Two main issues that can arise from chromosomal integration are the difficulty in inserting larger genes and the lower copy number of the gene of interest. This may lead to decreased production of the antigen, compared to a plasmid-based system. This could be counteracted by the use of a strong promoter used for high level expression of the gene of interest. The gene of interest does need to be inserted into a region of open chromatin that remains open for the majority of the bacterial cell cycle. The Dozois group has developed a system that inserts a gene of interest after *glmS* (432). *glmS* encodes L-Glutamine:D-fructose-6-phosphate aminotransferase (DFAT), an enzyme used in the first step of hexosamine metabolism that is constitutively active. While this system was originally developed to study *E. coli*, the same genes are present in *Salmonella*, allowing the system to be used in the development of *Salmonella* vectored vaccines. While we haven't seen any groups using CRISPR to develop heterologous antigen expressing *S.* Typhimurium strains, CRISPR technology can be successfully used in *Salmonella* and offers another method for chromosomal integration of short heterologous genes (433). With new tools at our disposal, we

are now able to ask questions about whether plasmids or chromosomal integration is the more suitable platform, however we hypothesize that the outcomes will vary depending on the heterologous antigen and the timing of its' delivery during infection.

With multiple groups working on developing *S.* Typhimurium vectored vaccines for different pathogens, we will need to address how previous vaccination or exposure to wild type *S.* Typhimurium affects the immune response to vaccination. People with a memory response to the vector may mount an ineffective response to the heterologous antigen expressed by the *Salmonella* vaccine strain, meaning they could only receive one *Salmonella*-vectored vaccine. This had been the leading hypothesis surrounding adenoviral vectored vaccines. However, during the COVID-19 pandemic, it has been demonstrated in clinical trials that a second dose of an adenoviral vectored vaccine can boost the immune response to the heterologous antigen (434). Metzger et al demonstrated in humans that priming with Ty21a and boosting with a heterologous antigen-expressing Ty21a led to detectable T cell responses to the heterologous antigen (435). However, there was no detectable humoral response, which is often a marker used by regulators to determine vaccine immunogenicity. This suggests that *S.* Typhimurium vaccines may have similar difficulty in stimulating responses to heterologous antigen after previous exposure. One of the benefits from these responses is perhaps a *S.* Typhimurium vectored vaccine could act as a "two disease" vaccine, and protect recipients from non-typhoidal *Salmonella* (NTS). In 2017 an estimated 95.1 million cases of enterocolitis caused by NTS occurred globally (436). NTS can in some cases also become invasive and there are mounting concerns about antibiotic resistant strains (437). A vaccine that could protect at risk populations from both NTS and a second pathogen would be highly beneficial.

One of the vital aspects of vaccine design is ensuring that a scaled-up manufacturing process is feasible. This is an advantage to using a *Salmonella* vectored vaccine. *Salmonella* is easy (and low cost) to grow, it doesn't require host cells for replication and has a short doubling time. In addition, the Ty21a vaccine only requires storage at 2-8ºC. Our lab has tested YS1646 and has found that lyophilised chromosomally integrated YS1646 is relatively stable for 3 months at room temperature (only a 10-fold decrease in viability). 2-8ºC storage with extended

stability at room temperature, is an easier cold chain to maintain compared to vaccines requiring either -20ºC or -80ºC storage.

A question that our lab is investigating is the use of *S.* Typhimurium vectored vaccines on their own compared to a multimodal vaccination strategy. We have seen that oral delivery of *S.* Typhimurium vectored vaccines does not generate a detectable IgG response in mice. Yet when delivered at the same time as an intramuscular dose of recombinant antigen, *S.* Typhimurium is able to increase the IgG titers compared to intramuscular vaccination alone. This may be particularly relevant as systemic antibody titers are often assessed by regulators to evaluate vaccine immunogenicity. While multimodal vaccination could still be delivered with one visit to a clinician, there are several downsides to this delivery method. The first being the loss of a needle-free vaccine, which has its obvious advantages. Secondly, the toxicity and safety testing of multimodal vaccines is more expensive and complicated as there are multiple components that need to be tested separately and in combination. While there is currently no multimodal vaccine on the market, during the COVID-19 pandemic we have seen increased flexibility with regulators. The CDC has authorized heterologous booster doses and this opens the possibility to the acceptance of a multimodal vaccine, if it is effective (438, 439).

1.11 *SALMONELLA* AND *CLOSTRIDIOIDES DIFFICILE*

Several groups have examined the possibility of using *S.* Typhimurium-derived flagellin as an adjuvant in *C. difficile* vaccine candidates. In 2011, Jarchum et al demonstrated that IP injection of FliC can protect mice from *C. difficile* pathogenesis, by maintaining the integrity of the intestinal epithelial barrier (440). Three doses of 15 μg of FliC delivered on -1, 0 and 1 dpi provides significant protection from lethal *C. difficile* challenge. This protection is dependent on TLR5. Treated mice have delayed *C. difficile* expansion and reduced IEC apoptosis. Subsequently, two groups have used *Salmonella* flagellin as an adjuvant in candidate *C. difficile* vaccines. Ghose et al vaccinated mice IP with a recombinant fusion protein of flagellin subunit D1 and the RBDs of TcdA or TcdB (441). The addition of the flagellin subunit D1 increases TcdA-specific IgA titers in the stool. Vaccination with and without the flagellin subunit D1 completely protects mice from lethal *C. difficile* challenge. The protection of mice from challenge with recombinant RBDs of TcdA or TcdB without adjuvants is consistent with

previous literature (345, 442). Recently, Wang et al developed a novel chimeric recombinant protein with the GT, CPD, RBDs of TcdA and TcdB and FliC from *S.* Typhimurium (443). Mice were vaccinated with 3 doses delivered IM. Vaccination elicits both IgG and IgA antibodies in the serum. The vaccine candidate protects mice from lethal NAP1 challenge, and the addition of FliC increases protection from IP toxin challenge. While these data are preliminary, they nonetheless suggest that the use of *S.* Typhimurium as a vaccine vector may be beneficial through TLR5 activation by the flagellin during vaccination.

1.12 RATIONALE AND RESEARCH OBJECTIVES

Despite the successes in transmission control, *Clostridioides difficile* still poses a major public health threat. The rate of CA-CDI is increasing and the size of the at-risk elderly/immunocompromised population is expected to double in the next few decades (13, 20, 289). First line treatment not only fails to cure a significant proportion of patients, but increased use of antibiotics is associated with antimicrobial resistance (AMR) (9, 444). AMR is widely considered to be one of the most serious global public health threats (445). Combatting AMR requires many approaches, but antibiotic stewardship and infection prevention are applicable in the health care setting (446). A vaccine against *C. difficile* would provide a key tool in the efforts to prevent CDI, leading to a decrease in the use of antibiotics and the associated risk of AMR. Unfortunately, over the course of the candidate's doctoral studies two vaccines have failed to meet their primary endpoint in phase III clinical trials (344, 347). Indeed, we had predicted that these vaccine candidates would not be entirely successful. Although targeting the toxins to prevent toxin mediated diseases has been a successful strategy, eliciting a systemic immune response against mucosal pathogen is counterintuitive (353, 354). For this reason, we developed a novel vaccine that targeted the induction of a mucosal response against the major *C. difficile* toxins.

The first objective of this thesis work was to develop antigen-expressing YS1646 strains that were capable of protecting mice from *C. difficile* challenge (**Chapter 2**). We developed 13 plasmid-based strains that expressed a portion of the RBD of TcdA or TcdB. Two were selected for *in vivo* testing. When delivered in a multimodal vaccination strategy, three doses delivered PO with a single dose of recombinant antigen delivered IM, they elicited both antigen-specific

IgG and IgA antibodies. These candidates provided 100% protection to mice from lethal *C. difficile* challenge. Having completed the proof-of-concept, we examined the longevity of the responses elicited by our vaccine candidates as a second objective (**Chapter 3**). We demonstrated that the IgG and IgA responses elicited by vaccination were maintained for 6 months in mice. All our vaccine strategies, including vaccination with only our antigen-expressing YS1646 strains provided significant protection from challenge 6 months after vaccination. Our third and final objective was to develop a stable vaccine candidate that would be appropriate for human use (**Chapter 4**). To this end, we developed six strains of YS1646 with the genes of the targeted RBD antigens chromosomally integrated (CI). We demonstrated that vaccination with our two selected candidates elicited a mucosal response as well as a systemic response. Oral vaccination against TcdA with the CI candidate completely protected mice from lethal challenge. The work outlined in this thesis is the candidate's contributions to the development of a novel, orally delivered *C. difficile* candidate vaccine. A proposed pathway to licensure for this vaccine candidate is described in **Chapter 5**.

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1.14 FIGURES AND LEGENDS

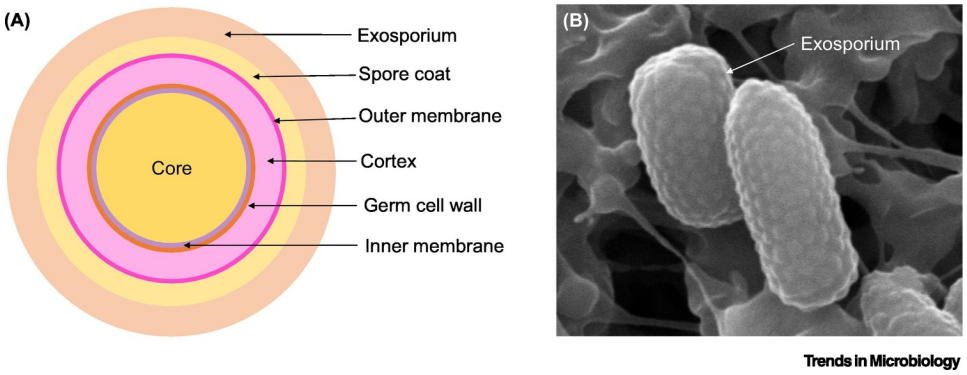

Figure 1.1 *Clostridioides difficile* Spore Structure. (A) Schematic cross-sectional representation of spore layer structure (not to scale). (B) Scanning electron microscopy image of the *C. difficile* strain NCTC 11204 spore surface showing the exosporium layer (image provided by Rachel Sammons, University of Birmingham).

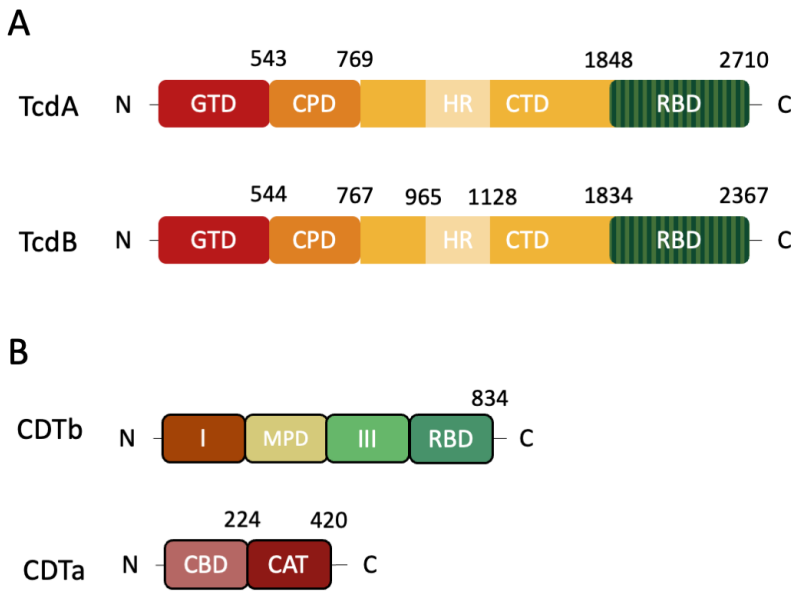

Figure 1.2 Domain Structure of *C. difficile* Toxins. A) The domain structure of Toxins A and B are shown, with the glucosyltransferase domain (GTD), cysteine protease domain (CPD), central translocation domain (CTD), with the hydrophobic region marked (HR), and the receptor binding domain (RBD). B) The domain structure of CDTb and CDTa are shown. CDTb has domain I, the membrane and pore formation domain (MPD), domain III and the RBD. CDTa has two identical domains with the CDTb binding domain (CBD) and the catalytic domain (CAT).

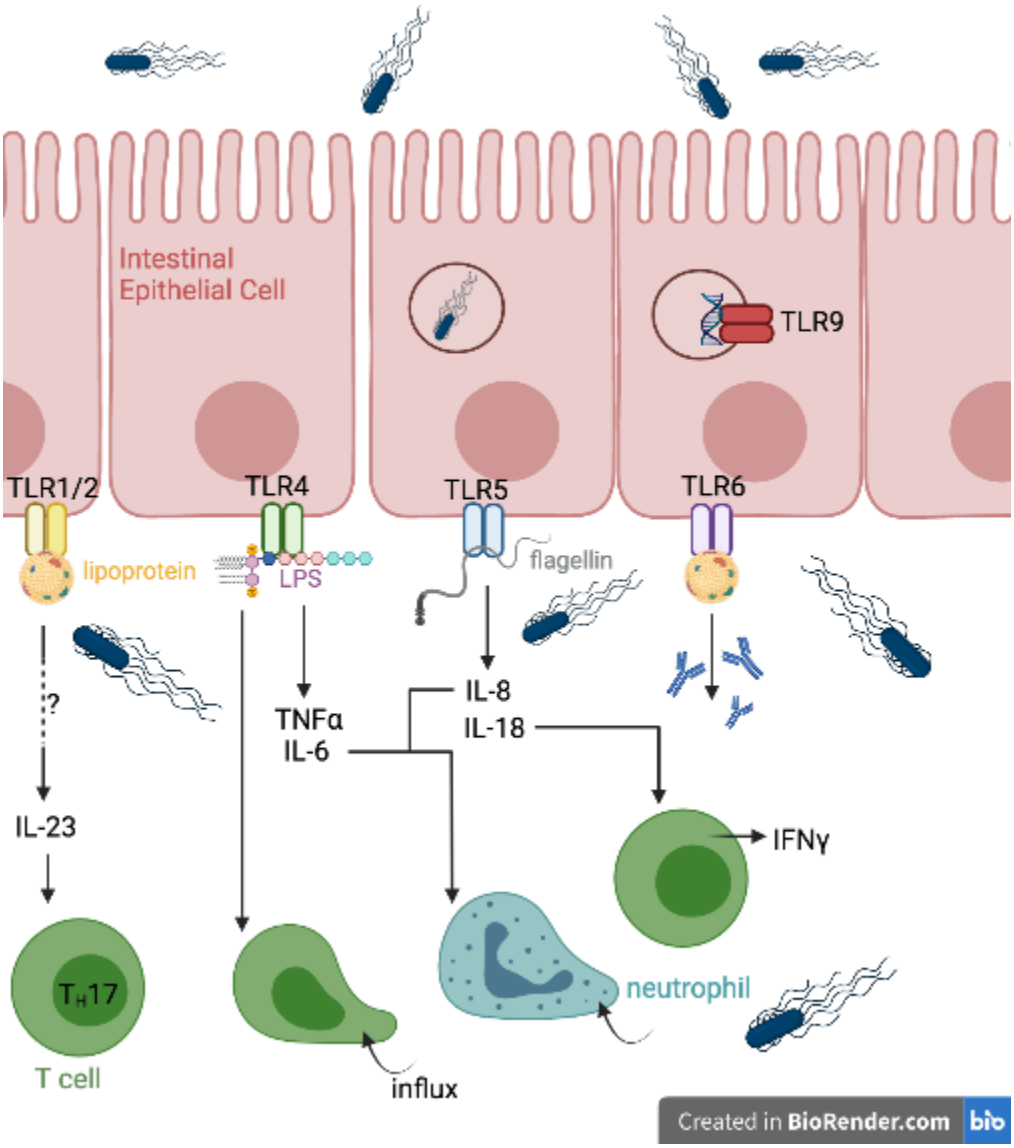

Figure 1.3 TLR driven responses to *Salmonella* Typhimurium infection. *Salmonella* Typhimurium has many different PAMPs that activate TLRs in the small intestine. Lipoproteins activate TLR1/2 and TLR6 on the basal membrane of IECs. TLR4 and TLR5 are activated by LPS and flagellin respectively. TLR9 is activated by CpG rich elements in *Salmonella* DNA present inside the IEC. TLR signalling drives an influx of T cells and neutrophils to the site of infection. Some signals push $CD4^+$ T cells towards T_H17 cells and others increase IFNγ production by T cells. TLR6 signalling leads to increased antibody production at the site of infection.

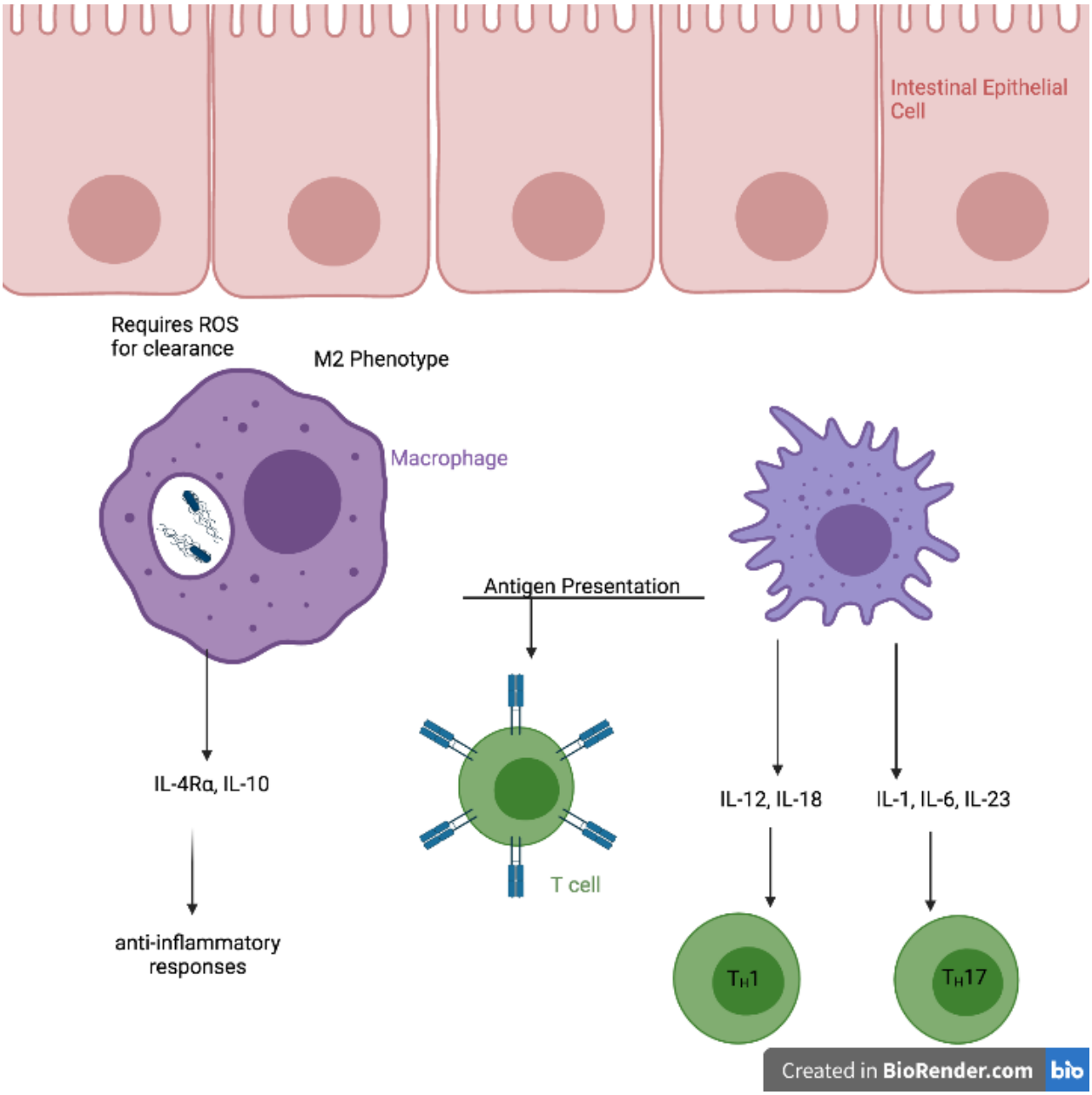

Figure 1.4 Macrophage responses to *Salmonella* Typhimurium infection in the lamina propria. Actively infected macrophages develop an M2 phenotype, secreting IL-4Rα and IL-10, generating anti-inflammatory responses. ROS is required for bacterial clearance from infected macrophages. Both actively infected and uninfected macrophages can participate in antigen presentation. Uninfected macrophages produce cytokines that induce T_H1 and T_H17 $CD4^+$ T cells.

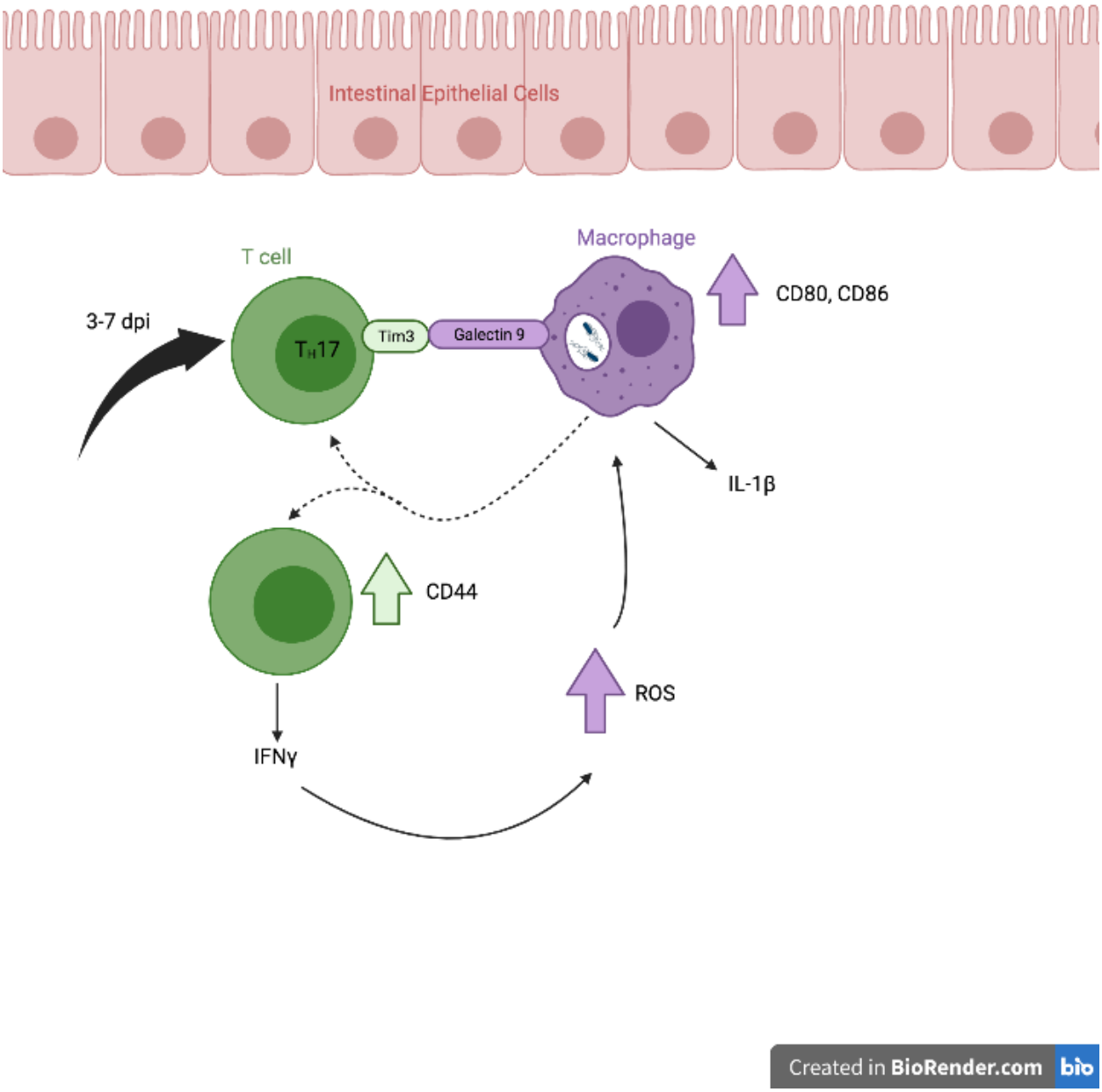

Figure 1.5 T cell responses to *Salmonella* Typhimurium infection in the small intestine. $CD4^+$ T cells influx into the gut 3-7 days post infection. They are initially T_H17 skewed and are capable of activating infected macrophages. With increased CD80 and CD86 expression macrophages are able to encourage CD44 upregulation and IFNγ production by $CD4^+$ T cells. IFNγ signalling increases ROS production in macrophages assisting in bacterial clearance.

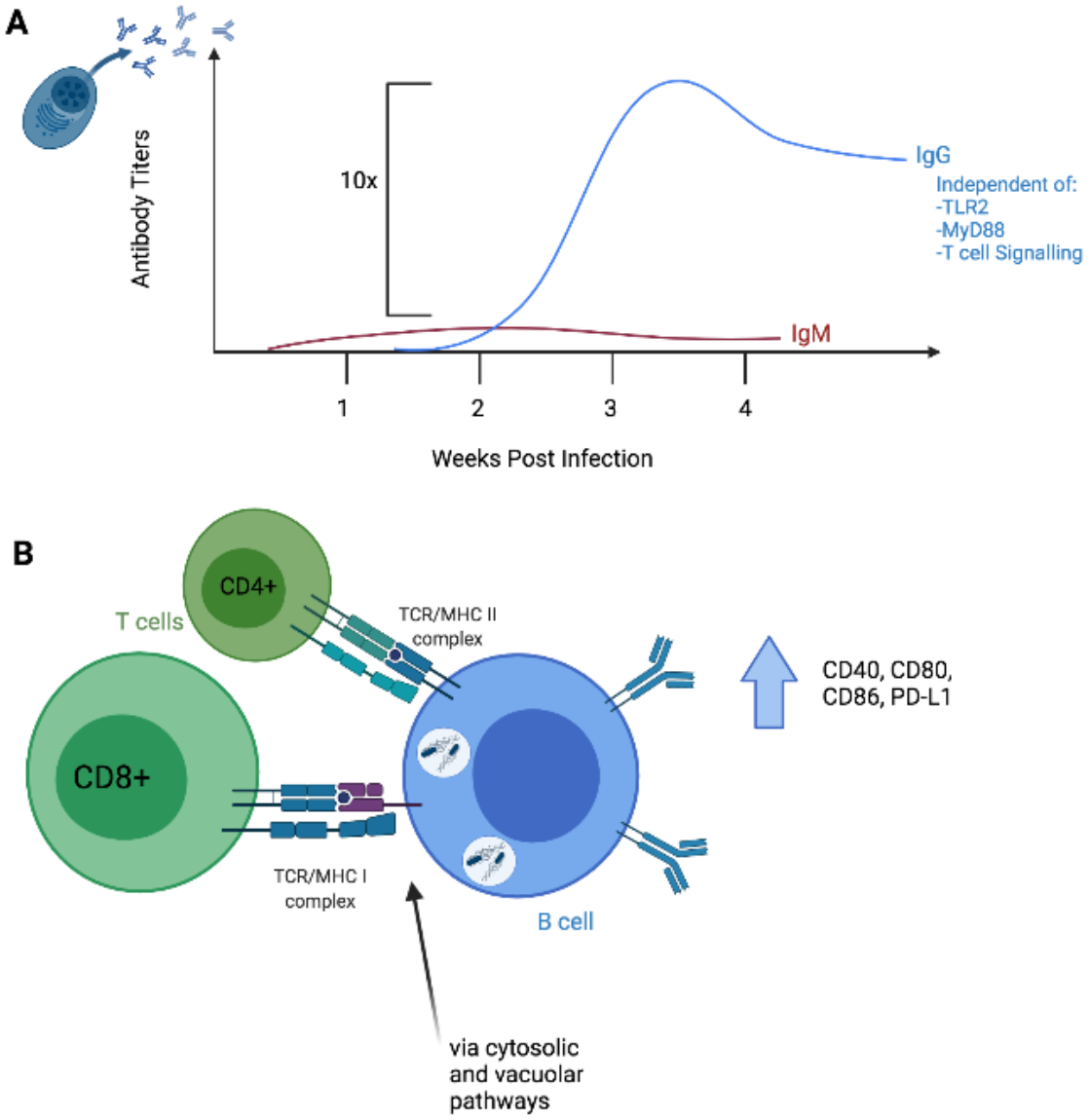

Figure 1.6 B cell responses to *Salmonella* Typhimurium infection. (A) IgM titers are detectable 4 dpi and peak 14 dpi in mice. IgG titers are delayed but can be up to 10x more expansive. The development of IgG against *S.* Typhimurium is independent of TLR2, MyD88 and T cell

signalling. (B) Infected B cells can also act as professional APCs. Through the cytosolic and vacuolar pathways, they can present to $CD8^+$ T cells. Antigen presentation to T cells increases expression of CD40, CD80, CD86 and PD-L1.

PREFACE TO CHAPTER 2

A vaccine is needed to reduce morbidity and mortality caused by *Clostridioides difficile*. All current strategies in development of a vaccine target the toxins and elicit a systemic immune response after multiple doses given across several months. We sought to develop a vaccine candidate that elicited a mucosal response against *C. difficile* and could generate a protective response in a shorter period of time. We had access to *Salmonella enterica* serovar Typhimurium YS1646, an attenuated strain that was originally developed as a cancer therapeutic and found safe in humans in a clinical trial. In this chapter, we repurposed YS1646 as a vaccine vector against *C. difficile*. We used a plasmid-based system for antigen expression and developed a multimodal vaccination strategy. We examined the immune response generated by our vaccine candidate and its protective efficacy.

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Chapter 2: Vaccination against *Clostridium difficile* using an attenuated *Salmonella* Typhimurium vector (YS1646) protects mice from lethal challenge

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2.1 ABSTRACT

Clostridium difficile disease is mediated primarily by toxins A and B (TcdA and TcdB). The receptor binding domains (RBD) of TcdA and TcdB are immunogenic and anti-RBD antibodies are protective. Since these toxins act locally, an optimal *C. difficile* vaccine would generate both systemic and mucosal responses. We have repurposed an attenuated *Salmonella enterica* serovar Typhimurium strain (YS1646) to produce such a vaccine. Plasmid-based candidates expressing either the TcdA or TcdB RBD were screened. Different vaccine routes and schedules were tested to achieve detectable serum and mucosal antibody titers in C57BL/6J mice. When given in a multimodality schedule over 1 week (day 0 IM+PO, days 2 and 4 PO), several candidates provided 100% protection against lethal challenge. Substantial protection (82%) was achieved with combined PO TcdA/TcdB vaccination alone (d0, 2 and 4). These data demonstrate the potential of the YS1646-based vaccines for *C. difficile* and strongly support their further development.

2.2 INTRODUCTION

Clostridium difficile is one of the most important nosocomial pathogens in the world (1,2). Clinically-apparent *C. difficile* infection (CDI) is most often caused by antibiotics that disrupt the gastrointestinal microbiota, permitting overgrowth of *C. difficile* and production of toxins A and B (TcdA and TcdB). TcdA, an enterotoxin, and TcdB, a cytotoxin, represent two of the principal virulence factors of *C. difficile* and both are expressed by most clinical isolates (3). Together, they disrupt the actin cytoskeleton of enterocytes in the gastrointestinal epithelium, resulting in fluid accumulation, inflammation and severe tissue damage (4). Some strains of *C. difficile* produce an additional toxin called the binary toxin or CDT (5).

The prevalence and severity of CDI has increased significantly in most countries over the past 2-3 decades (2,6). More than 370,000 cases occur every year in North America alone with an estimated total cost exceeding 6 billion dollars (7). Currently, antibiotics are routinely recommended for the treatment of CDI (eg: metronidazole, vancomycin, fidaxomicin alone or in combination) despite the irony of treating a disease caused by antibiotics with further antibiotics. Recurrent CDI after treatment and severe CDI are significant problems that are poorly-responsive to antibiotics (8). Effective control of CDI is complicated by asymptomatic carriage, including post-treatment, and by spores that can persist in the environment for prolonged periods.

Preventing CDI-associated morbidity and mortality requires new approaches including the development of vaccines. *Clostridium difficile* is non-invasive, so CDI is largely a toxin-mediated disease. Indeed, the outcome of CDI in both animal models and humans is strongly correlated with the host antibody response to TcdA and/or TcdB (9). These toxins have therefore been a major focus of both active and passive immunotherapeutic strategies and several toxin-based vaccines have advanced to phase II/III clinical trials (10). Of particular interest to the current studies, both pre-clinical and clinical-stage work support the idea of targeting the RBDs of these toxins (11-13). Whether whole protein, toxoid or RBD however, most of the effort to elicit anti-toxin responses has focused on peripheral, intramuscular (IM), administration of these antigens. Furthermore, as is typical for non-living vaccines, these candidates require an adjuvant and multiple doses over several months to achieve an adequate immune response (10).

Several groups have demonstrated the potential of oral vaccines to elicit protective responses to RBDs in animal models of CDI. For example, Guo et al demonstrated that oral administration of *Lactococcus lactis* expressing both the RBDs of TcdA and TcdB could elicit both IgA and IgG and protect mice from lethal challenge (14). In conceptually similar studies, Hong and colleagues showed that hamsters given *Bacillus subtilis* spores expressing the carboxy-terminal segment of TcdA orally ($TcdA_{26-39}$) can be protected from *C. difficile* colonization by mucosal IgA (15). We considered that a locally-invasive but highly attenuated *Salmonella enterica* serovar Typhimurium vector might be even more effective in the induction of local and systemic anti-RBD responses. The flagellin protein of *S.* Typhimurium has been proposed as a general mucosal adjuvant through its action on toll-like receptor (TLR)-5 (16). Ghose & colleagues have shown that the *S.* Typhimurium flagellin protein (Flic) fused to TcdA or TcdB can elicit toxin-specific IgA and IgG and protect mice from lethal challenge (17). Other *Salmonella* products such as lipopolysaccharide (LPS) would be expected to further enhance immune responses by triggering additional pathogen recognition receptors (PRRs: TLR4) (18). Live attenuated *Salmonella* have other potential advantages as vaccine vectors including targeting of intestinal M cells that overlie the gut-associated lymphoid tissues (GALT) and invasion of macrophages leading to the induction of both humoral and cellular responses to their foreign protein 'cargo' (19, 20). They also have a large 'carrying' capacity and are easy to manipulate both in the laboratory and at industrial scale.

In recent years, live attenuated *Salmonella* has been increasingly used to express foreign antigens against infectious diseases and cancers (21-23). *Salmonella enterica* is a facultative intracellular pathogen that replicates in a unique membrane-bound host cell compartment, the *Salmonella*-containing vacuole (12). Although this location limits exposure of both *Salmonella* and foreign proteins produced by the bacterium to the immune system, the organism's type III secretion systems (T3SS) can be exploited to translocate heterologous antigens into the host cell cytoplasm. *Salmonella enterica* encodes two distinct T3SS within the *Salmonella* pathogenicity islands 1 and 2 (SPI-I and SPI-II) that become active at different phases of infection (24). The SPI-I T3SS translocates effector proteins upon first contact of the bacterium with epithelium cells through to the stage of early cell invasion. In contrast, SPI-II expression is induced when

the bacterium has been phagocytosed. Several effector proteins translocated by these T3SSs have been tested in the promotion of heterologous antigen expression in *Salmonella*-based vaccine development programs but how effector protein-mediated secretion of heterologous antigens affects immune responses is still poorly understood (23, 25). Although there is considerable experience in using the attenuated *S. typhi* vaccine strain (Ty21a: Vivotif™) in the delivery of heterologous antigens, we chose to use *S.* Typhimurium YS1646 as our candidate vector (22). This strain, originally named VNP20009, is attenuated by mutations in its msbB (LPS) and purI (purine biosynthesis pathway) genes and was originally developed as a tumour targeting vector (26). With a major investment from Vion Inc, YS1646 was carried through pre-clinical and toxicity testing in rodents, dogs and non-human primates before a phase I clinical trial where it ultimately failed (27). More recently, YS1646 has been used to express a chimeric *Schistosoma japonicum* antigen in a murine model of schistosomiasis (28). Repeated oral administration of one of the engineered strains elicited a strong systemic IgG antibody response, induced antigen-specific T cells and provided up to 75% protection against *S. japonicum* challenge.

In the current work, we exploited constitutive promoters and T3SS-specific promoters and secretory signals to generate 15 YS1646 strains with plasmid-based expression of the RBD portion of either TcdA or TcdB. These strains were screened for protein expression in monomicrobial culture and RAW 264.7 murine macrophages. The most promising constructs were advanced to immunogenicity testing in adult female C57BL/6 mice using different routes (eg: recombinant protein IM, YS1646 strains orally (PO)) and schedules (eg: repeat dosing, multimodality, prime-pull) to achieve the best serologic response in the shortest period of time. Two of the YS1646 strains elicited strong systemic IgG responses and provided up to 100% protection from lethal challenge when administered in a multimodality schedule over 5 days (IM + PO on day 0 followed by PO boosting on days 2 and 4).

2.3 RESULTS

2.3.1 Transformed *S.* Typhimurium YS1646 expresses heterologous antigen

Plasmids expressing the RBDs of Toxin A (rbdA) or Toxin B (rbdB) under the control of different promoters and secretory signals were constructed (Fig 2.1). The promoter-secretory signal combinations included SPI-I- (eg: SopE2, SptP) and SPI-II-specific pairings (eg: SseJ,

SspH2) as well as pairings used by both SPI-I and SPI-II secretory pathways (eg: SteA, SteB, SspH1). Some of the secretory signals were also paired with constitutively active or inducible promoters nirB, pagC, and lac (Table 2.1). All primers used in the study are listed in Supplemental Table 2.1. A set of plasmids with the same promoter/secretory signal pairings but expressing enhanced green fluorescent protein (EGFP) were also constructed. All plasmids were transformed into *S.* Typhimurium YS1646.

Using the EGFP-expressing strains, we screened for antigen expression in monomicrobial culture and during *in vitro* infection of murine RAW 264.7 macrophages. Most strains produced detectable EGFP in monomicrobial culture (summarized in Supplemental Table 2.2). The YS1646 candidates were readily macropinocytosed and a fluorescent signal was detected for all of the EGFP expressing strains (Fig 2.2a). Expression varied considerably between strains however with the strongest signal driven by the pagC_SspH1_EGFP construct. Some constructs (eg: SspH2_SspH2_EGFP) had good initial EGFP expression but survival and/or replication in the macrophages was markedly reduced at 24 hours post-infection.

Expression of the targeted *C. difficile* RBDs in monomicrobial culture and murine macrophages was examined by Western blotting at 1 and 24 hours post-infection. Modest production of rbdA and rbdB could be documented by most strains in monomicrobial culture, but very few strains had detectable antigen expression during macrophage infection (rbdA in Fig 2.2b; rbdB in Fig 2.2c; summarized in Supplemental Table 2.2). For example, the pagC_SspH1 pairing drove strong expression of both antigens in broth and at 1-hour post-infection in the murine macrophages but the SspH2_SspH2 pairing failed to drive detectable rbdB expression and the level of rbdA production was barely detectable only in monomicrobial culture. Secretion of the RBDs into extracellular medium was examined in monomicrobial culture (Supplemental Table 2.2). Only pagC_SspH1_rbdA had detectable antigen secretion. The lack of secretion detection may be due to low levels of expression in the cells.

The most promising constructs were advanced to mouse immunogenicity testing. Since neither monomicrobial culture nor RAW 264.7 cells are adequate models for the low oxygen

tension and poly-microbial environment of the gastrointestinal tract, we included some of the apparently negative constructs in the *in vivo* immunogenicity testing.

2.3.2 rbdA and rbdB delivered by YS1646, in combination with recombinant rbdA/rbdB, is highly immunogenic in mice

Using the rapid induction of serum antigen-specific IgG as our principal screening tool, a multimodal schedule was identified as the most promising vaccination strategy. This schedule was comprised of a single IM dose of the recombinant RBD (rrbd) on day 0 with 3 PO doses of the corresponding RBD-expressing strain on days 0, 2 and 4. When sera were collected 3-4 weeks after vaccination using this schedule, rbdA-specific (Fig 2.2d) and rbdB-specific (Fig 2.2e) IgG titers were consistently elevated. IgG responses generated were consistently higher than those achieved by recombinant antigen delivered IM and pQE_null strain delivered PO but these differences did not reach statistical significance (P= 0.1727 for rbdB). In contrast, mice that received only the three PO doses of YS1646 strains bearing the RBD antigens had no detectable serum IgG response. Despite the failure to induce IgG with PO vaccination, three doses of YS1646 on alternate days could nonetheless prime for a significant response to a subsequent IM booster dose delivered 3 weeks later (data not shown). Both the multimodal and oral only vaccination schedules generated higher rbdA- (Figure 2.2f) and rrbdB-specific IgA (Figure 2.2g) levels in the intestinal tissues than delivering recombinant antigen intramuscularly although the differences did not reach statistical significance with the relatively small number of animals used in these experiments. Interestingly, mice vaccinated against only one toxin tended to have higher IgA antibodies against that toxin, than mice vaccinated against both toxins, raising the possibility of some degree of antigen interference.

2.3.3 Selection of Candidate YS1646 Strains for Challenge Testing

The combined screening studies identified two YS1646 constructs that were carried forward into challenge testing (pagC_SspH1_rbdA and SspH2_SspH2_rbdB) (Supplemental Table 2.2). Since oral immunization generated intestinal IgA (Figure 2.2f,g) and was able to prime animals for a strong systemic IgG response to a subsequent IM boost (data not shown), we included PO-only groups in challenge studies in addition to the multimodality IM+PO schedule.

2.3.4 YS1646-vectored rbdA and rbdB vaccines protect mice from lethal *C. difficile* challenge

5 weeks after vaccination, mice were challenged with a lethal dose of *C. difficile* vegetative cells and monitored for weight loss, clinical score and death. Overall, 67% of the PBS control group succumbed to infection between 36 and 72 hours post-infection (Figure 2.3a). Only 18% of mice that received three PO doses of the pagC_SspH1_rbdA and SspH2_SspH2_rbdB strains, succumbed to the infection. All other vaccinated groups had 100% survival (Figure 2.3a). The recovery of animals that survived appeared to be complete: surviving mice recovered their original body weight. Mice were followed for up to 3 weeks after infection and no relapses were observed. During infection, mice were 'clinically' scored 1-3 times daily (Fig 2.3b). Although the group vaccinated with rrbdA + rrbdB IM and the pQE_null strain PO had 100% survival, 71% of these mice were severely ill: achieving a score of 12 or higher (14 = animal care cut-off for humane endpoint). The proportion of severely ill mice in groups that received any antigen-expressing YS1646 strain with an IM dose of recombinant protein was consistently much lower (0% - 14%). None of the animals in the group that received rrbdB IM plus three doses of the SspH2_Ssph2_rbdB strain PO experienced severe illness. All mice had very low or completely normal clinical scores by 6 days post infection. There is a strong negative correlation between serum anti-rbdB IgG, both before and after challenge, and the highest clinical score achieved by individual mice (Figure 2.2e; Supplemental Figure 2.1b,d; Supplemental Table 2.3). Our results suggest that in our mouse model, an immune response directed towards TcdB is sufficient to obtain effective protection from *C. difficile* challenge.

The combined IM+PO schedules also elicited small but detectable increases in antigen-specific IgA levels in the intestinal tissues after challenge although the increase only reached statistical significance for the animals vaccinated against rbdB alone ($P<0.05$ versus the control group) (Supplemental Figure 2.1c,d). Interestingly, the intestinal anti-rbdB IgA levels tended to be slightly lower in the animals that received both of the YS1646 constructs PO compared to those vaccinated only against rbdB (Figure 2.2f, Supplemental Figure 2.1d) although this difference also failed to reach statistical significance.

2.4 DISCUSSION

The pathology associated with CDI is thought to be toxin-mediated and there are strong precedents for the efficacy of vaccine-induced anti-toxin antibodies in the prevention or modification of toxin-mediated diseases (eg: tetanus, diphtheria, cholera) (3, 29, 30). Indeed, an anti-TcdB monoclonal antibody (bezlotoxumab or Zinplava™: Merck) has recently been shown to reduce the frequency of recurrent *C. difficile* disease (31). In addition to passive immunotherapy, the generation of anti-toxin antibodies is also the predominant strategy being pursued by both large and small pharmaceutical companies with an interest in developing *C. difficile* vaccines (10). However, the most advanced of these candidate vaccines require multiple doses of antigen with an adjuvant over several months to achieve high serum antibody concentrations (10,32). Furthermore, even though CDI is a disease of the gastrointestinal mucosa, none of these candidates would be expected to generate an effective mucosal immune response. Both in theory and as demonstrated in the current work, the delivery of the same *C. difficile* toxin antigens using a live attenuated *S.* Typhimurium vector has the potential to induce both local and systemic immunity. There are several groups working on delivering *C. difficile* antigen at the mucosal surface (15, 33). Recently, Wang et al used a non-toxigenic *C. difficile* to target TcdB and TcdA, through expression of the RBDs (33). They found that after 3 doses delivered every two weeks, that their vaccine candidate was effective at protecting mice and hamsters. In this study we provide proof of concept that a multimodality vaccination schedule using a single IM dose of recombinant toxin A and/or toxin B receptor binding domain proteins with PO delivery of YS1646 bearing the same RBD antigens over a five-day period can rapidly induce both systemic and mucosal responses and protect mice from an otherwise lethal challenge. Although the amount of IgA present in the intestinal tissues after vaccination was relatively low after YS1646 vaccination, the induction of an effective local immune response by these vaccines was strongly supported by the fact that oral vaccination alone provided substantial protection despite the absence of detectable serum antibodies prior to challenge.

Although logistically more complicated and considered ‘inelegant’ by some, heterologous prime-boost and multimodality vaccination strategies are gaining traction for a wide range of infections and other complex conditions, such as cancers (34-36). Of particular interest to the current proposal, such combined modality approaches have shown promise in

eliciting effective immune responses against mucosal pathogens such as HIV/SHIV and influenza (35, 36). Combined modality strategies may also have a place in toxin-mediated diseases in which high titres of preformed antibodies are needed such as *Clostridium perfringens* infection or when a rapid but sustained response is desirable such as Ebola (37, 38). These new approaches have the potential to enhance the character, kinetics and durability of the response (39). While simpler vaccination strategies will certainly be carried forward as our candidate vaccines advance into larger animal models, toxicity testing and ultimately clinical trials, the multimodality method we developed in the murine model would be relatively easy to administer to the 'typical' person who might benefit from a *C. difficile* vaccine: ie: those in or entering a long-term care facility or being prepared for elective surgery (40-42). Only one face-to-face clinic/office visit would be needed to receive the IM vaccine and the first (supervised) PO vaccine after which the remaining two PO doses on alternate days could be taken autonomously (as is currently the practice for the live attenuated *S. typhi* Ty21a vaccine). The long clinical experience with Ty21a also confirms the feasibility of delivering an attenuated *Salmonella* to the intestinal tissues (22). Although such a rapid vaccination schedule would likely increase compliance, it is also possible that the durability of the response would be compromised (43). Clearly, long-term follow-up studies will be needed to more completely evaluate the optimal vaccination strategy for the YS1646 vaccine candidates.

While still early in development, there are certainly safety concerns in potentially exposing elderly or debilitated individuals to a live attenuated bacterium as a vaccine vector. Several of the immunological and physiological factors that put the elderly at risk for *C. difficile* also make a live attenuated vaccine that targets the gut mucosa a potential risk. Even though wild-type *S.* Typhimurium typically causes only mild disease localized to the gastrointestinal tract in humans, it can sometimes cause invasive disease with serious outcomes (22, 44). The YS1646 strain that is the backbone of our vaccine platform carries mutations of both an LPS gene (*msbB*) and a part of the purine production machinery (*purI*) that render it highly attenuated (27). Although the mechanisms of attenuation differ, the live attenuated Ty21a *S. typhi* vaccine has an excellent safety record, even in elderly subjects (22). In the critical development pathway of YS1646 as a possible anti-cancer agent in the early 2000s, this strain proved to be safe in multiple small (eg: mice, rats) and large animal models (eg: dogs, *Rhesus macaques*) (D.

Bermudes, unpublished data) before it was permitted to advance to a phase 1 clinical trial (27). In this trial, a single dose of up to $3x10^8$ colony-forming units (cfu) of YS1646 was administered intravenously to 24 subjects with metastatic melanoma or renal cell carcinoma without any major safety signals. Most of the subjects in this trial cleared YS1646 from their blood stream in <12 hours (27). It was subsequently suggested that an unexpected susceptibility of YS1646 to physiologic levels of CO_2 present in human tissues (~5%) may have contributed to its failure as a cancer therapy (45). In contrast to the need for YS1646 to disseminate and replicate actively in tumour tissues as an anti-cancer agent, to be an effective vaccine vector, YS1646 only needs to invade locally and express the targeted antigen for a short period of time in the GALT (22). Of course, a necessary step prior to the use of YS1646 as a candidate *C. difficile* vaccine will be chromosomal integration of the most promising TcdA and TcdB RBD constructs: this work is currently underway. Although chromosomal integration will reduce the copy number of our target gene and therefore protein expression, we will try to mitigate these effects through the use of strong promoters (eg: PpagC) and the integration of tandem repeats for both antigens. Since several of our current candidates were able to elicit immune responses despite undetectable antigen production *in vitro*, we are optimistic that we will be able to design chromosomally integrated strains that are immunogenic. To our knowledge, the only other clinical experience with attenuated *S.* Typhimurium is that of Hindle et al who exposed a small number of human subjects to a single oral dose of up to $1x10^9$ cfu of a strain bearing aroC and SPI-II T3SS mutations without dissemination or ill effects (46). Hindle et al observed asymptomatic shedding of an attenuated *S.* Typhimurium strain for 3 weeks in the feces of patients, with all shedding ending by week 4 after vaccination. Although YS1646 has different attenuating mutations and may have a different colonization profile in humans after oral delivery, asymptomatic persistence of this *S.* Typhimurium strain was also demonstrated for at least 1 week in a small proportion of subjects after intravenous delivery in the early anti-cancer phase 1 trial (27). While we acknowledge that the question of colonization/persistence will eventually need to be addressed with regulators should a YS1646-vectored *C. difficile* vaccine enter into clinical trials, the mere fact of persistence does not automatically disqualify a vaccine candidate. Indeed, several of the live attenuated vaccines on the market are routinely shed by vaccinees for longer than a week. These include rotavirus that is shed for up to 9 days post vaccination, measles that can be

detected for at least 14 days, oral polio that can persist for several months and varicella that causes a lifelong latent infection (47-50).

This study has several limitations. First, there is no perfect small or large animal model for human CDI (40,41). Although mice are widely considered to be one of the most informative models, mice are also the natural host for *S.* Typhimurium. Indeed, *S.* Typhimurium infection in mice is commonly used as a model for human typhoid fever caused by S. Typhi (51). As a result, the degree to which an 'attenuated' *S.* Typhimurium such as YS1646 will have a similar profile of attenuation in mice and humans is unknown. Indeed, although mice remained completely healthy during and after oral vaccination, we observed colonization of the spleen and liver by some of the YS1646 strains carrying either TcdA or TcdB constructs for 1-2 weeks after vaccination (data not shown). Although we do not expect to see such dissemination in humans due to the CO_2 sensitivity of YS1646, it is certainly possible that immunity generated in response to persistent antigen expression over days-weeks will differ from that induced by a shorter exposure. Such persistence may not occur in other models such as the gnotobiotic piglet that has been used as a large animal model for *C. difficile* infection (40, 41). Second, the relative sensitivity of different animals used in *C. difficile* studies and humans to the major *C. difficile* toxins is not fully consistent (52). Nonetheless, it is likely that both TcdA and TcdB contribute to pathology in the mouse model we are using and in humans (53). As a result, we are optimistic that our findings in the murine model will predict outcomes in humans and our goal is to develop a YS1646-based vaccine that can provide protection against both TcdA and TcdB. Finally, the choice of optimal promoter-secretory signal pairings for *in vivo* expression of the RBD antigens is complicated by our inability to truly reflect the conditions to which the YS1646 strains will be exposed in the human gastrointestinal tract and the GALT. We have tried to mitigate this risk by using a multi-layered screening process but acknowledge that we have already identified constructs that do not appear to produce the targeted RBD *in vitro* (in monomicrobial culture or RAW 264.7 cells) but still elicit strong antibody responses in the mouse model.

In this work, we describe the repurposing of a live attenuated *S.* Typhimurium strain (YS1646) as a vaccine-vector to target the major toxins of *C. difficile*. Administered in a 5-day, multimodality schedule (IM x 1, PO x 3), these candidate vaccines elicited high serum IgG titres

and provided complete protection from lethal challenge in a mouse model. This proof-of-concept study supports the further development of these candidate vaccines by chromosomal integration of the two most promising constructs (SspH2_Ssph2_rbdB and pagC_SspH1_rbdA), evaluation in the gnotobiotic piglet model and toxicity testing (40, 41). If these next steps are successful, a phase I human study with a 'mixed' TcdA/TcdB vaccine will be pursued.

2.5 METHODS

2.5.1 Bacterial Strains and Growth Conditions

Salmonella enterica Typhimurium YS1646 (ΔmsbB2 ΔpurI ΔSuwwan xyl^-; ATCC 202165: ATCC, Manassas, VA) was obtained from Cedarlane Labs (Burlington, ON). *Escherichia coli* DH5α (ThermoFischer Scientific, Eugene, OR) was used for production of recombinant plasmids. Plasmids were introduced into *E. coli* or YS1646 by electroporation (2 μg of plasmid at 3.0kV, 200 Ω, and 25 μF; GenePulser XCell, Bio-Rad, Hercules, CA, USA). Transformed bacteria were grown in Luria Broth (LB) with 50 μg/mL of ampicillin (Wisent, St. Bruno, QC) for cells containing plasmids with the pQE_30 backbone.

Clostridium difficile Strain VPI 10463 (ATCC 43255) was obtained from Cedarlane Labs and used for challenge experiments. Cells were maintained in meat broth (Sigma-Aldrich, St Louis, MO) containing 0.1% (w/v) L-cysteine (Sigma-Aldrich) in an anaerobic jar. For colony counts, *C. difficile* containing media was serially diluted and streaked onto pre-reduced Brain Heart Infusion (BHIS) plates (BD Biosciences, Mississauga, ON), containing 0.1% (w/v) L-cysteine. Plates were left to grow at 37°C in an anaerobic jar for 24h.

2.5.2 Plasmid Construction

2.5.2.1 Vaccine Candidate plasmids

The pQE_30 plasmid backbone containing an ampicillin resistance gene used for antigen expression in the vaccine candidates was cloned from the plasmid roGFP_IL_pQE30, a gift from David Ron (Addgene, plasmid #48633) (54). PCR was used to obtain the SopE2, SptP, SseJ, SspH1, SspH2, SteA and SteB promoter and secretory signal sequences from YS1646. The pagC promoter from YS1646 and the nirB promoter from *E coli* were also PCR amplified. The lac

promoter was incorporated into the 5' PCR primer. The antigenic C-terminal ends of the receptor binding domains for Toxin A ($TcdA_{1820-2710}$) and Toxin B ($TcdB_{1821-2366}$) were amplified by PCR from *C. difficile* VPI 10463. Restriction sites were incorporated 5' of the promoters (Xho1), between the secretory signal and the antigen (Not1), and at the 3' end of the antigen sequence (AscI) (Figure 2.1). Primers used are listed in Supplemental Table 2.1. DNA sequencing confirmed that plasmids had the expected sequence (McGill University Genome Centre, Montreal, QC). EGFP antigen was cloned from the plasmid pEGFP_C1 (Clontech, Mountain View, CA) with the Not1 and Asc1 incorporated in the primers. All plasmids are named based on the promoter, secretory signal and antigen used, these are described in Table 2.1. The unedited pQE_30 plasmid was transformed into YS1646 as a control and is referred to as pQE_null.

2.5.2.2 Recombinant TcdA and TcdB expression

Protein expression and purification of recombinant $TcdA_{1820-2710}$ (rbdA) and $TcdB_{1821-2366}$ (rbdB) was accomplished using the pET-28b plasmid (Novagen, Millipore Sigma, Burlington, MA), with an Isopropyl-β-D-1-thiogalactopyranoside (IPTG) inducible promoter and kanamycin resistance gene. A 6x His tag and stop codon was added at the 3' end. The expression vector was transformed into *E. coli* C2566l (New England BioLabs, Whitby, ON) as above. Transformed bacteria were grown in a 37° shaking incubator with 30 μg/ml of kanamycin (Wisent), until the absorbance at 600 nm (OD_{600}) reached 0.5-0.6. IPTG (Invitrogen, Carlsbad, CA) was then added and expression was induced for 3-4 hours. Cells were pelleted by centrifugation at 3000xg for 10 minutes at 4°C. Cells were lysed, and lysate was collected and purified using Ni-NTA affinity chromatography (Ni-NTA Superflow by Qiagen, Venlo, Limburg, Netherlands). The eluate was analyzed by Coomassie blue staining of polyacrylamide gels and Western Blot using a monoclonal antibody directed against the His-tag (Sigma-Aldrich).

2.5.3 Macrophage Infection

RAW 264.7 cells (ATCC TIB-71) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Wisent) supplemented with 10% fetal bovine serum (FBS), penicillin (100 000 U/mL), and streptomycin (100μg/mL; Wisent); cells were passaged when they reached ~90% confluence. For each passage, cells were washed with Hank's Balanced Salt Solution (HBSS) without calcium and magnesium (Wisent) and detached from the flasks using 0.25% Trypsin

(Wisent). RAW 264.7 cells were seeded in Falcon™ Polystyrene 12-well plates (Corning Inc., Corning, NY) at a density of $1x10^6$ cells/well for infection experiments 24 hours later. RAW 264.7 cells were infected at a multiplicity of infection (MOI) of either 40 or 100. For western blotting, cells were then incubated at 37ºC in 0% CO_2, as YS1646 is sensitive to increased CO_2 levels. Infection was allowed to proceed for an hour then cells were washed 3x with PBS and resuspended in DMEM with 50 μg/mL of gentamicin (Wisent) was added, to kill extracellular YS1646. After 2 hours, the gentamicin concentration was lowered to 5 μg/mL.

2.5.3.1 Fluorescence (EGFP) Microscopy

RAW 264.7 cells, plated on 8-well microscope chamber-slides (Eppendorf, Hamburg, Germany) at $1.8x10^5$ cells/chamber, were infected at a MOI of 40 with YS1646 strains transformed with the EGFP constructs. Infected cells were incubated at 37ºC in 5% CO_2. 24 hours after infection, cells were stained with 4',6-diamidino-2-phenylindole (DAPI; ThermoFischer Scientific) and fixed with 4% paraformaldehyde (Sigma Aldrich). A Zeiss LSM780 laser scanning confocal microscope was used for imaging (405nm laser for excitation of DAPI, 488nm laser for excitation of EGFP) and acquisition and processing was performed using ZEN software (Zeiss, Toronto, ON).

2.5.3.2 Western Blot

For antigen expression in monomicrobial culture, transformed YS1646 strains were grown overnight in LB with 50 μg/mL of ampicillin at 37ºC and 0% CO_2, centrifuged at 21 130xg for 10 minutes, resuspended in PBS, then mixed in with NuPAGE Lithium Dodecyl Sulfate (LDS) sample buffer (Invitrogen) according to the manufacturer's instructions. For antigen expression in RAW 264.7 macrophages, infection was allowed to proceed for either 1 hour or 24 hours. Samples were then collected, centrifuged, resuspended in PBS, and mixed with sample buffer as above. All samples were heated for 10 min at 70°C, then cooled on ice. Proteins were separated on a 4-12% Bis-Tris Protein Gel (Invitrogen) and transferred to nitrocellulose membranes using the Trans-Blot® Turbo™ RTA Mini Nitrocellulose Transfer Kit (Bio-Rad, Hercules, CA). For detection of $TcdA_{5458-8130}$ and $TcdB_{5461-7080}$, the membranes were incubated first with anti-Toxin A chicken IgY (1:5000; Abnova, Taipei, Taiwan) and anti-Toxin B chicken IgY antibodies (1:10,000; Abnova), respectively followed by goat anti-chicken IgY conjugated

to horseradish peroxidase (1:10,000; ThermoFisher Scientific). Immunoreactive bands were visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) and autoradiography film (Denville Scientific, Holliston, MA).

2.5.4 Mice

6 to 8-week-old female C57BL/6J mice were obtained from Charles River Laboratories (Montreal, QC) and were kept in pathogen-free conditions in the Animal Resource Division at the McGill University Health Center Research Institute (RI-MUHC). All animal procedures were approved by the Animal Care Committee of McGill University and performed in accordance with the guidelines of the Canadian Council on Animal Care.

2.5.4.1 Vaccination

For oral vaccinations, mice were gavaged with $1x10^9$ cfu of YS1646 strains in 0.2ml of PBS (days 0, 2 and 4). When both strains were given, $5x10^8$ cfu of each strain was used, for a total of $1x10^9$ cfu of YS1646 given in 0.2ml of PBS. Intramuscular (IM) injections contained a total of 10 μg of recombinant protein and 250 μg of Alhydrogel (alum; Brenntag BioSector A/S, Frederikssund, Denmark) in 50 μL administered into the *gastrocnemius* muscle using a 28G needle.

2.5.4.2 Blood and Intestine sampling

Baseline serum samples were collected from the lateral saphenous vein prior to all other study procedures using microtainer serum separator tubes (Sarstedt, Nümbrecht, Germany). Serum samples were also collected at the end of the study by cardiac puncture in mice after isoflurane/CO_2 euthanasia. Serum separation was performed according to manufacturer's instructions and aliquots were stored at -20ºC until used. At study termination, 10 cm of the small intestine, starting at the stomach, was collected. Intestinal contents were removed and the tissue was weighed and stored in a Protease Inhibitor (PI) Cocktail (Sigma Aldrich – P8340) at a 1:5 dilution (w/v) on ice until processed. The tissue was homogenized (Homogenzier 150; Fisher Scientific, Ottawa, ON), centrifuged at 2500xg at 4ºC for 30 minutes and the supernatant was collected. Supernatants were stored at -80ºC until analyzed by ELISA. For post-challenge data, samples were collected from survivors 3 weeks after infection.

2.5.4.3 *Clostridium difficile* Challenge

C. difficile challenge experiments were performed essentially as described by Warren and colleagues (55, 56). Briefly, mice were pre-adapted to acidic water by adding acetic acid at a concentration of 2.15 μL/mL [v/v] to their drinking water one week prior to antibiotic treatments. Six days prior to infection, an antibiotic cocktail included 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), colistin (0.042 mg/mL; Sigma Aldrich) was added to the drinking water. After 3 days, regular water was returned and 24 hours prior to infection, mice received clindamycin (32 mg/kg; Sigma Aldrich) intraperitoneally in 0.2mL of PBS using a 28G needle. Fresh *C. difficile* cultures were used in our challenge model so the dose used was estimated on the day of infection based on OD_{600} values and the precise inoculum was calculated 24 hours later. This procedure led to the use of different *C. difficile* doses in the two challenge studies performed ($1.7x10^7$ or $1.97x10^5$ cfu/mouse). The challenge dose was delivered by gavage in 0.2ml of meat broth culture media. Mice were then monitored and scored 1-3 times daily for weight loss, activity, posture, coat quality, diarrhea and eye/nose symptoms (56). Mice with a score of 14/20 or above and/or with ≥20% weight loss were considered at a humane endpoint and were euthanized. Any mouse found dead, was given a score of 20. Survivors were followed and euthanized approximately 3 weeks after infection.

2.5.5 Antibody Quantification

Whole toxin A (List Biologicals, Campbell, CA) or recombinant rbdB were used to coat U-bottom high-binding 96-well ELISA plates (Greiner Bio-one, Frickenhausen, Germany). A standard curve was included on each plate using mouse IgG antibodies (Sigma Aldrich) or mouse IgA antibodies (Sigma Aldrich). Plates were coated with 50 μL of Toxin A (1.0 μg/mL), rbdB (0.25 μg/mL) or IgG/IgA standards overnight at 4°C in 100 mM bicarbonate/carbonate buffer (pH 9.5). Wells were washed with PBS 3x then blocked with 150μL of 2% bovine serum albumin (BSA; Sigma Aldrich) in PBS-Tween 20 (0.05%; blocking buffer; Fisher Scientific) for 1 hour at 37ºC. Serum samples were heat-inactivated at 56ºC for 30 minutes before a 1:50 dilution in blocking buffer. Intestinal supernatants were added to the plates neat. All sample dilutions, including standard curve dilutions, were assayed in duplicate (50 μL/well). Plates were

incubated for 1 hour at 37ºC then washed 4x with PBS prior to the addition of either HRP-conjugated anti-mouse total IgG antibodies (75 μL/well at 1:20 000 in blocking buffer; Sigma Aldrich) or HRP-conjugated anti-mouse IgA antibodies (75 μL/well at 1:10 000 in blocking buffer; Sigma Aldrich). Plates were incubated for 30 minutes (IgG) or 1 hour (IgA) at 37ºC. Six washes with PBS were performed before the addition of 100 μL/well of 3,3',5,5'-tetramethyl benzidine (TMB) detection substrate (Millipore, Billerica, MA). Reactions were stopped after 15 minutes with 50 μL/well of 0.5 M H_2SO_4. Plates were read at 450 nm on an EL800 microplate reader (BioTek, Instruments Inc., Winooski, VT). The concentration of antigen-specific antibodies in each well (ng/mL) was estimated by extrapolation from the standard curve.

2.5.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software. For analysis of antibody titers, one-way non-parametric Kruskal-Wallis ANOVA was performed with Dunn's multiple comparison analysis comparing all groups. Statistical significance was considered to have been achieved when $P \leq 0.05$. Data are presented as means ± standard deviation (SD) or means ± standard error of the mean (SEM). For analysis of survival, the log rank (Mantel-Cox) test was used to compare all groups to the PBS control group. The Bonferroni method was used to correct for multiple comparisons. In Supplemental Table 2.3, correlations are based on Spearman's r coefficient (non-parametric), 95% Confidence Intervals were calculated, and two tailed p-values were determined.

2.6 DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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2.8 CONTRIBUTIONS

KW, LX and BW designed the study and experiments. LX and KW designed, constructed and validated the plasmids. KW performed the animal experiments. AK performed experiments under the supervision of KW. KW and BW performed analysis on the data and prepared the manuscript.

2.9 COMPETING INTERESTS

The authors have no competing interests to declare.

2.10 MATERIALS AND CORRESPONDENCE

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2.12 TABLES

Table 2.1. Plasmids used in this study

Plasmid	Promoter	Secretory Signal	Antigen
pQE_null	---	---	---
pSopE2_SopE2_rbdB	SopE2	SopE2	$TcdB_{1821-2366}$
pSseJ_SseJ_rbdB	SseJ	SseJ	$TcdB_{1821-2366}$
pSptP_SptP_rbdB	SptP	SptP	$TcdB_{1821-2366}$
pSspH1_SspH1_rbdB	SspH1	SspH1	$TcdB_{1821-2366}$
pSspH2_SspH2_rbdB	SspH2	SspH2	$TcdB_{1821-2366}$
pSteA_SteA_rbdB	SteA	SteA	$TcdB_{1821-2366}$
pSteB_SteB_rbdB	SteB	SteB	$TcdB_{1821-2366}$
ppagC_SspH1_rbdB	pagC	SspH1	$TcdB_{1821-2366}$
pSspH2_SspH2_rbdA	SspH2	SspH2	$TcdA_{1820-2710}$
plac_SopE2_rbdA	lac	SopE2	$TcdA_{1820-2710}$
plac_SspH1_rbdA	lac	SspH1	$TcdA_{1820-2710}$
pnirB_SopE2_rbdA	nirB	SopE2	$TcdA_{1820-2710}$
pnirB_SspH1_rbdA	nirB	SspH1	$TcdA_{1820-2710}$
ppagC_SopE2_rbdA	pagC	SopE2	$TcdA_{1820-2710}$
ppagC_SspH1_rbdA	pagC	SspH1	$TcdA_{1820-2710}$

2.13 FIGURES AND LEGENDS

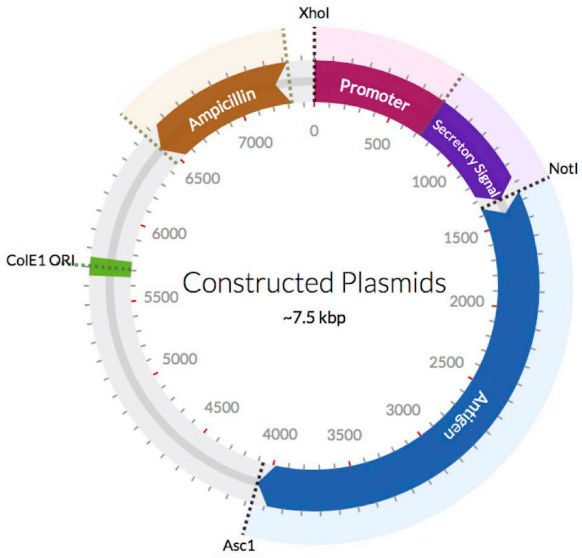
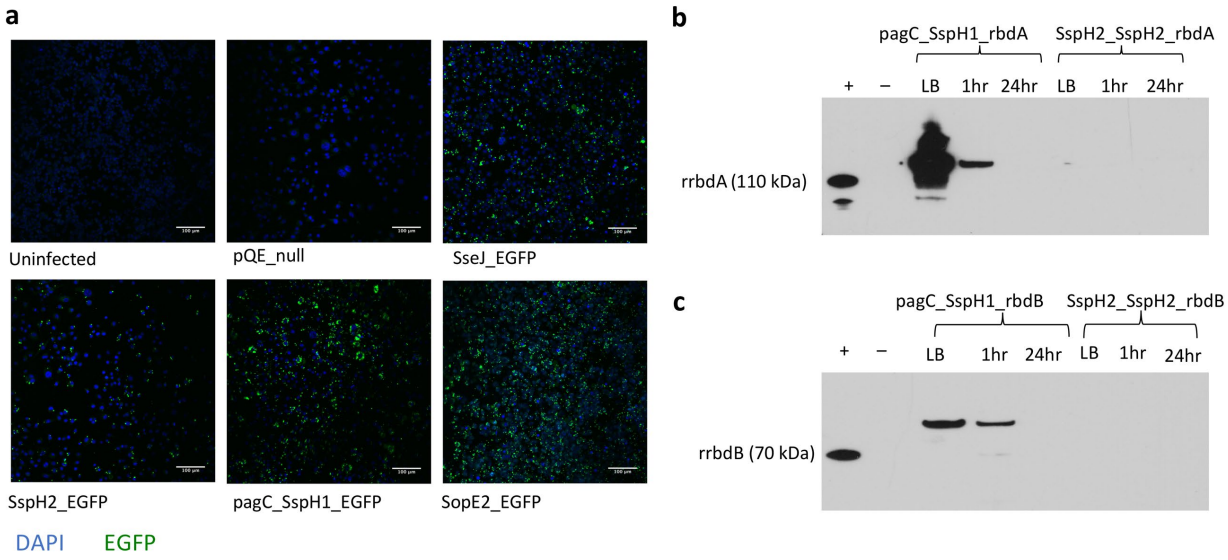
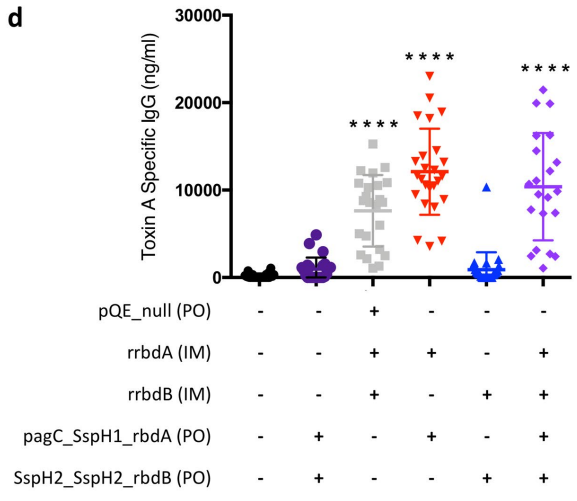
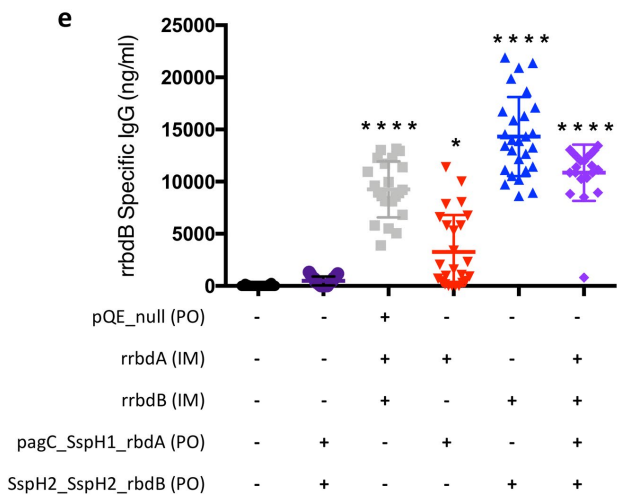
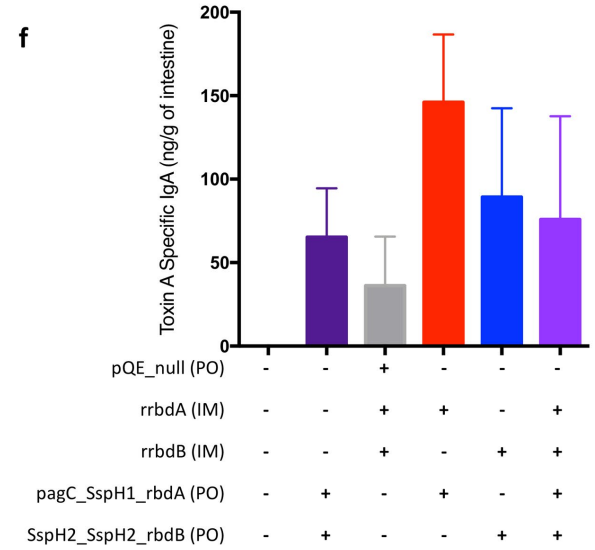


Figure 2.1 Generic plasmid map: the pQE_30 plasmid containing an ampicillin resistance gene was used as the backbone. The promoter and secretory signals were inserted between XhoI and NotI sites. The antigen sequence was inserted between NotI and AscI sites. Plasmids were between 3.4 kbp (pQE_null), and 7.5 kbp in size.


a
Uninfected
pQE_null
SseJ_EGFP
SspH2_EGFP
pagC_SspH1_EGFP
SopE2_EGFP
DAPI
EGFP
b
pagC_SspH1_rbdA
SspH2_SspH2_rbdA
+ – LB 1hr 24hr LB 1hr 24hr
rrbdA (110 kDa)
c
pagC_SspH1_rbdB
SspH2_SspH2_rbdB
+ – LB 1hr 24hr LB 1hr 24hr
rrbdB (70 kDa)


d
Toxin A Specific IgG (ng/ml)
30000
20000
10000
0
****
****
****
pQE_null (PO) - - + - - -
rrbdA (IM) - - + + - +
rrbdB (IM) - - + - + +
pagC_SspH1_rbdA (PO) - + - + - +
SspH2_SspH2_rbdB (PO) - + - - + +


e
rrbdB Specific IgG (ng/ml)
25000
20000
15000
10000
5000
0
****
*
****
****
pQE_null (PO) - - + - - -
rrbdA (IM) - - + + - +
rrbdB (IM) - - + - + +
pagC_SspH1_rbdA (PO) - + - + - +
SspH2_SspH2_rbdB (PO) - + - - + +


f
Toxin A Specific IgA (ng/g of intestine)
200
150
100
50
0
pQE_null (PO) - - + - - -
rrbdA (IM) - - + + - +
rrbdB (IM) - - + - + +
pagC_SspH1_rbdA (PO) - + - + - +
SspH2_SspH2_rbdB (PO) - + - - + +

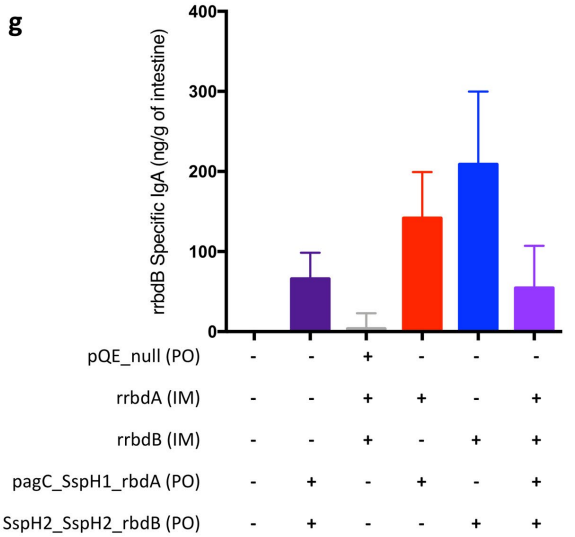

g
rrbdB Specific IgA (ng/g of intestine)
400
300
200
100
0
pQE_null (PO) - - + - - -
rrbdA (IM) - - + + - +
rrbdB (IM) - - + - + +
pagC_SspH1_rbdA (PO) - + - + - +
SspH2_SspH2_rbdB (PO) - + - - + +

Figure 2.2 Transformed YS1646 strains expressed heterologous antigen. (**a**) EGFP expressing strains of YS1646 were added to RAW 264.7 macrophages *in vitro* and visualized 24 hours later using a fluorescent microscope. Images are representative of two repeats. Antigen expression was examined by western blot from YS1646 strains transformed with rbdA (**b**) and rbdB (**c**) plasmids. Samples were collected after 16 hours of growth in LB and 1h and 24h after infection of RAW 264.7 macrophages. Gels were run with a positive control (recombinant RBD antigen, without secretion signals, produced in *E. coli*) and film was exposed for 2 minutes. The increased size of the RBDs produced in YS1646 are consistent with the secretion signals that are not cleaved. Mice were immunised with a dose of 10μg recombinant antigen (rrbdA and/or rrbdB) intramuscularly, and three doses of $1x10^9$ cfu of antigen expressing YS1646 (pagC_SspH1_rbdA and/or SspH2_SspH2_rbdB), orally every other day. Serum was collected 3-4 weeks after vaccination and Toxin A-specific IgG (**d**) and rrbdB-specific IgG (**e**) were detected by ELISA (n = 21-28, 4 repeats). Data are presented as mean and standard deviation (SD). Intestines were collected 5 weeks after vaccination and Toxin A-specific IgA (**f**) and rrbdB-specific IgA (**g**) were detected by ELISA (n = 4-5, one repeat). Data are presented as PBS subtracted mean and standard error of the mean (SEM). Kruskal-Wallis test and Dunn's Multiple Comparison test were used to compare between all groups. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ compared to the PBS control group.

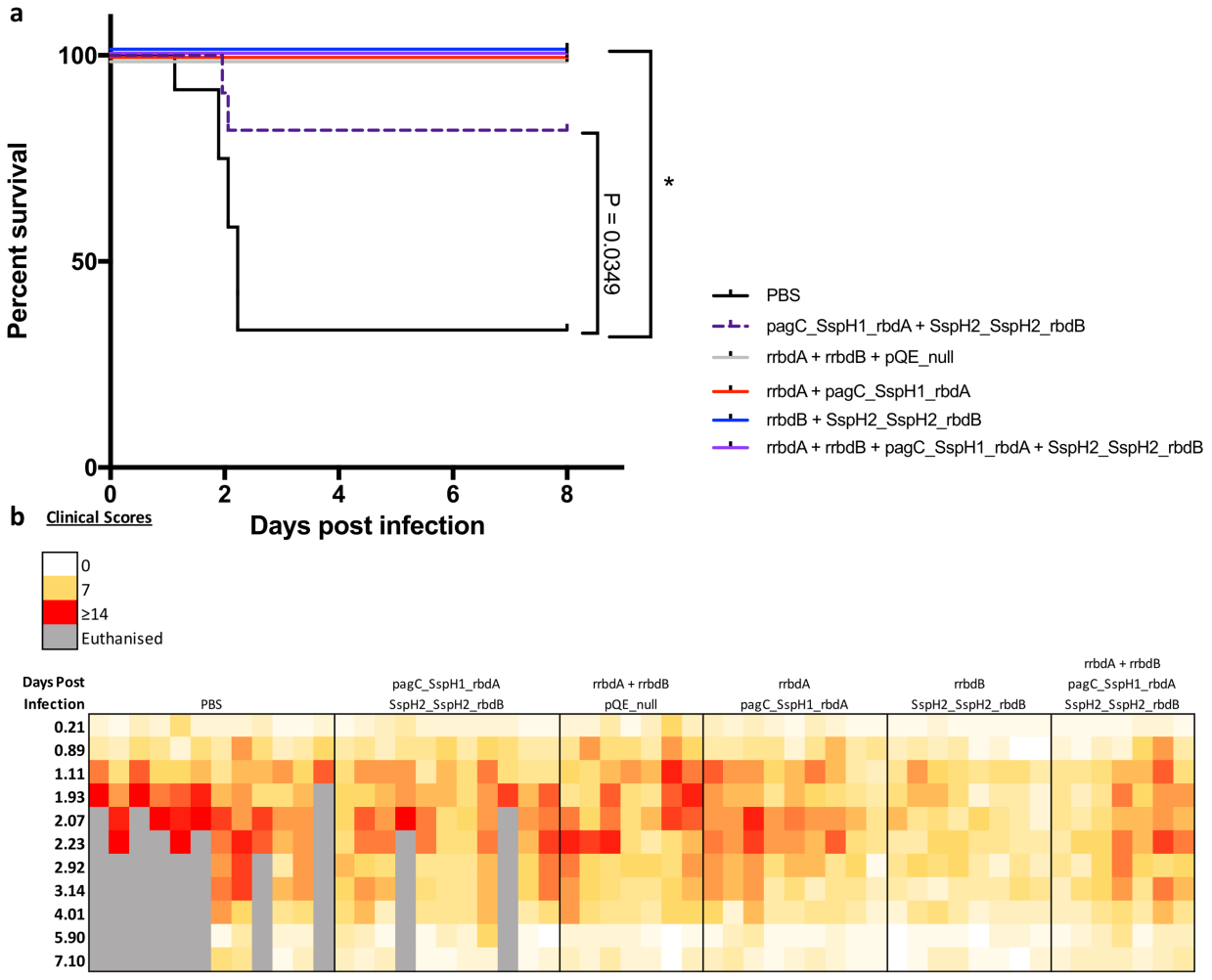


Figure 2.3 Vaccination with receptor binding domain (rbd) antigens protected against *C. difficile* challenge. Mice were immunised with a dose of 10µg of recombinant antigen (rrbdA and/or rrbdB) intramuscularly, and three doses of $1x10^9$ cfu of antigen expressing YS1646 (pagC_SspH1_rbdA and/or SspH2_SspH2_rbdB), orally every other day. 5 weeks after vaccination, mice were challenged PO with freshly cultured *C. difficile* ($1.97x10^5$ cfu and $1.70x10^7$ cfu). Mice were clinically scored 1-3 times daily by a blinded observer. A score of ≥14/20 and/or >20% loss of the starting body weight were considered humane endpoints. Survival (**a**) and clinical scores (**b**) are shown (n= 7-12, 2 repeats). Log rank (Mantel-Cox) test was used to compare all groups to the PBS control group. Correction of the p value for multiple comparisons was done using the Bonferroni method. * $P< 0.01$ compared to the PBS control group.

2.14 SUPPLEMENTAL FIGURES AND LEGENDS

Supplemental Table 2.1. Primers used in plasmid construction

	Forward Primer (5' --> 3')	Reverse Primer (5' --> 3')	Source
SopE2 promoter and secretory signal	CCGCTCGAGTAAAAATGTTCCTCG ATAAA	CATGGTAGTTCTCCTTTTAG	YS1646
SptP promoter and secretory signal	CGCCTCGAGTTTACGCTGACTCAT TGG	CATTTTTCTCTCCTCATACTTTA	YS1646
SseJ promoter and secretory signal	CGCCTCGAGACATAAAACACTAGC ACT	CGCCTCGAGACATAAAACACTAG CACT	YS1646
SspH1 promoter and secretory signal	CGCCTCGAGCGCTATATCACCAAA AC	CTCTGCGGCCGCGGTAAGACCTG ACGCTC	YS1646
SspH2 promoter and secretory signal	CGCCTCGAGGTTTGTGCGTCGTAT	CTCTGCGGCCGCATTCAGGCAGG CACGCA	YS1646
SteA promoter and secretory signal	CGCCTCGAGGTTTCGCCGCATGTT G	CTCTGCGGCCGCATAATTGTCCA AATAGT	YS1646
SteB promoter and secretory signal	CGCCTCGAGCGCTCCAGCGCTTCG A	CTCTGCGGCCGCTCTGACATTAC CATTT	YS1646
Lac promoter	CGCCTCGAGCATTAGGCACCCCAG GCTTTACACTTTATGCTTCCGGCTC GTATGTTGTGTGGAATTGTGAGCG GATAA, GTGGAATTGTGAGCGGATAACAAT TTCACACAGGAAACAGCTATGACC ATGACTAACATAACACTATCCAC		Sequence is in the primers
nirB promoter	CGCCTCGAGTTGTGGTTACCGGCC CGAT	CGCGCGGCCGCCGGATCTTTACT CGCATTAC	DH5α *E. coli*
pagC promoter	CGCCTCGAGGTTAACCACTCTTAA TAA	AACAACTCCT TAATACTACT	YS1646
SopE2 Secretion Signal	GGCGGTAATAGAAAAGAAATCGA GGCAAAAATGACTAACATAACACT ATCCAC	AAGTCGCGGCCGCCGGATCTTTA CTCGC	YS1646
SspH1 Secretion Signal	GGCGGTAATAGAAAAGAAATCGA GGCAAAAATGTTTAATATCCGCAA TACACAACCTT	CTCTGCGGCCGCGGTAAGACCTG ACGCTC	YS1646

rbdA	CGCGCGGCCGCGACTTATTACTATGAT	TAGTCGGCGCGCCCGCCATATATCCCAGG	VPI 10463
rbdB	CCGGCGGCCGCAGAGAAATTTTATATTAAT	AGTCGGCGCGCCGTTCACTAATCACTAATTG	VPI 10463
EGFP	CGCGCGGCCGCGGTGAGCAAGGGCGAG	AGTCGGCGCGCCTTACTTGTACAGCTCGTC	pEGFP_C1

Primers used to replicate the sequences from source DNA are shown. Some sequences were further edited to include an ATG start site between the promoter and secretory signal.

Supplemental Table 2.2. *In vitro* and *in vivo* screening of plasmids

	In vitro						*In vivo* (IM Prime, PO Boost)			
	EGFP Detection (EGFP expressing strains)		Antigen Detection by WB				Serum IgG		Intestinal IgA	
Strains	LB	RAW 264.7 (24h)	LB	Secretion in LB	RAW 264.7 (1hr)	RAW 264.7 (24h)	rbdB	rbdA	rbdB	rbdA
pQE_null	0	0	0	0	0	0	0	0	0	0
SopE2_SopE2_rbdB	+++	+++	+	0	0	0	0	<ctl	0	<ctl
SseJ_SseJ_rbdB	+	++	+	0	0	0	++	<ctl	0	0
SptP_SptP_rbdB	+	+	+	0	+	0	+	<ctl	+	0
SspH1_SspH1_rbdB	+	+	0	0	0	0	++	<ctl	++	0
SspH2_SspH2_rbdB	++	++	0	0	0	0	+++	<ctl	+++	0
SteA_SteA_rbdB	+++	++	+	0	0	0	++	<ctl	+++	0
SteB_SteB_rbdB	+	+++	0	0	0	0	<ctl	<ctl	0	0
pagC_SspH1_rbdB	n/a	+++	+	n/a	+	0	n/a	n/a	n/a	n/a
SspH2_SspH2_rbdA	++	++	0	n/a	0	0	n/a	n/a	n/a	n/a
lac_SopE2_rbdA	+	+	+	0	0	0	<ctl	<ctl	0	0
lac_SspH1_rbdA	0	0	0	0	0	0	<ctl	<ctl	0	0
nirB_SopE2_rbdA	++	+	+	0	+	0	<ctl	<ctl	0	++
nirB_SspH1_rbdA	++	+++	+	0	+	0	<ctl	<ctl	0	0
pagC_SopE2_rbdA	n/a	++	0	0	0	0	<ctl	<ctl	0	+++
pagC_SspH1_rbdA	n/a	+++	+	+	+	0	<ctl	<ctl	0	+++

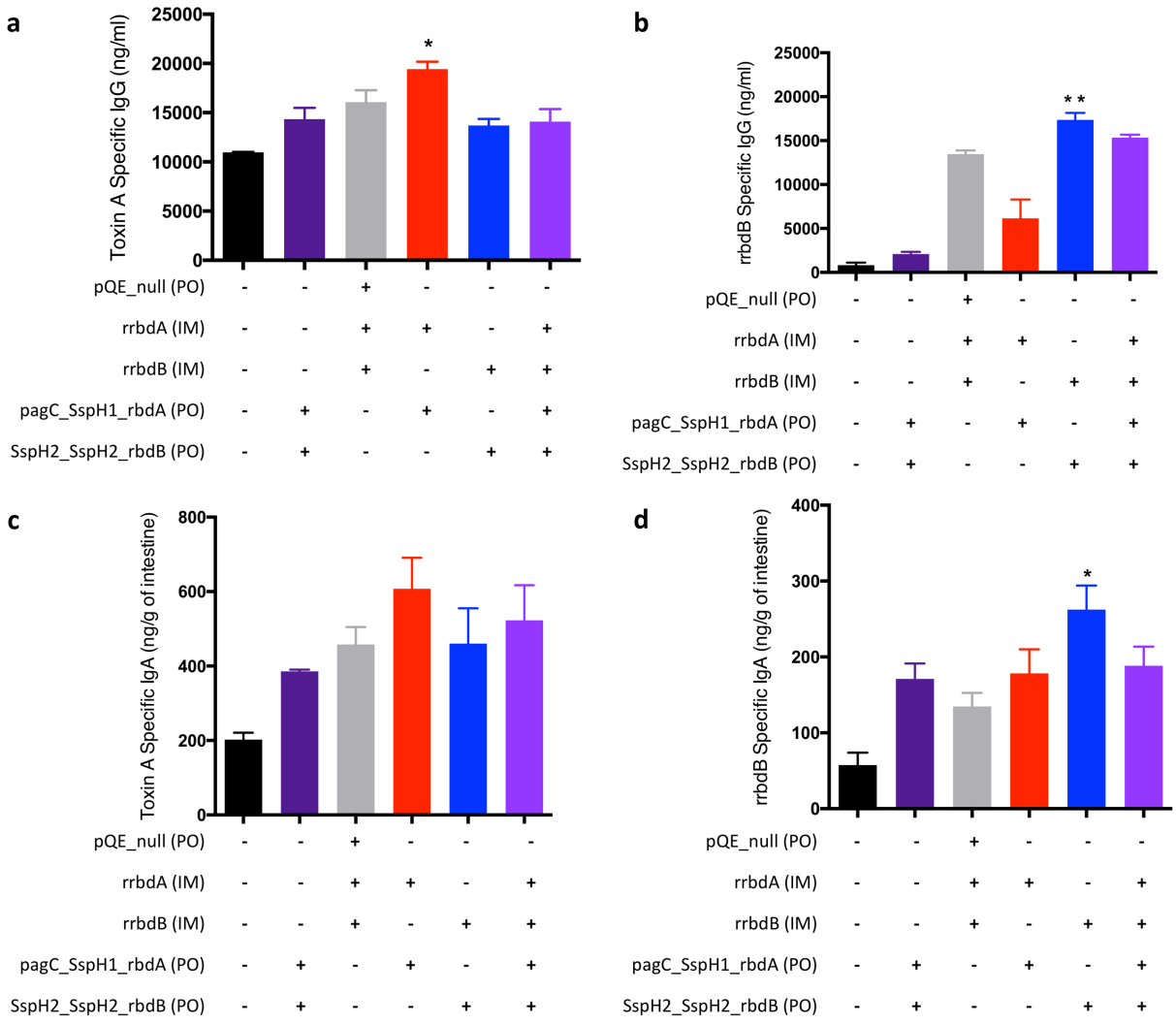
EGFP detection is based on the EGFP expressing strains with the same promoter and secretory signal as the listed strain. Strains that were not assessed are indicated in the table as "n/a". Detection by Western blot is designated as either antigen is detected "+" or not "0". For *in vivo* screening, mice were vaccinated with 10ug of protein IM (rbdA/rbdB) adjuvanted with alum and three weeks later the response was boosted by the YS1646 strains given by PO in 3 doses (n=2-4 mice/group). Serum and intestines were collected 3 weeks after the boost. Titers are shown compared to the control group of the listed protein delivered IM, boosted with pQE_null strain of YS1646. Titers lower than the control are listed as "<ctl". Titers that match the control are listed

as “0”. Titers higher than the control were divided into three categories; “+”, “++”, “+++” with increasing mean titers.

Supplemental Table 2.3. Correlations between antibody titers and clinical scores

		Tox A IgG Pre	Tox A IgG Post	Tox A IgA Post	rbdB IgG Pre	rbdB IgG Post	rbdB IgA Post	Salmonella IgG Pre	Salmonella IgG Post
Mean Score (all)	p value	ns	ns	ns	****	****	**	*	ns
	r	/	/	/	-0.735	-0.6555	-0.5047	-0.4031	/
	95% CI	/	/	/	(-0.8554, -0.5392)	(-0.8189, -0.3939)	(-0.7278, -0.1849)	(-0.6433, -0.09067)	/
Mean Score (vax)	p value	ns	ns	ns	**	**	**	/	/
	r	/	/	/	-0.7191	-0.6744	-0.6708	/	/
	95% CI	/	/	/	(-0.9031, -0.3124)	(-0.8857, -0.2318)	(-0.8843, -0.2257)	/	/
Highest Score (all)	p value	ns	ns	ns	****	***	**	*	ns
	r	/	/	/	-0.7177	-0.6068	-0.5158	-0.4031	/
	95% CI	/	/	/	(-0.8453, -0.5128)	(-0.7904, -0.3234)	(-0.7348, -0.1994)	(-0.6433, -0.09067)	/
Highest Score (vax)	p value	ns	*	*	ns	ns	*	*	/
	r	/	0.5643	0.6453	/	/	-0.6238	-0.3741	/
	95% CI	/	(0.0008807, 0.8558)	(0.1283, 0.8865)	/	/	(-0.8653, -0.1475)	(-0.6288, -0.05671)	/

Correlations are based on Spearman's r coefficient (non-parametric), 95% Confidence Intervals were calculated, and a two tailed p value was determined. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$



Supplemental Figure 2.1 Vaccination with antigen expressing YS1646 increases post-challenge antibody titers in the sera and intestines of survivors. Mice were immunised with a dose of 10µg recombinant antigen (rrbdA and/or rrbdB) intramuscularly, and three doses of $1x10^9$ cfu of antigen expressing YS1646 (pagC_SspH1_rbdA and/or SspH2_SspH2_rbdB), orally every other day. 5 weeks after vaccination, mice were challenged with $1.7x10^7$ cfu of *C. difficile*. 3 weeks after infection, serum and intestines were collected from survivors. Post-challenge serum toxin A specific IgG antibodies (**a**) and rrbdB specific IgG antibodies (**b**) were detected by ELISA. Intestinal toxin A specific IgA antibodies (**c**) and rrbdB specific IgA antibodies (**d**) were detected by ELISA (n= 2-8, one experiment). Mean and standard error of the mean (SEM) are shown. Kruskal-Wallis test and Dunn's Multiple Comparison test were used to compare between all groups. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 compared to the PBS control group.

PREFACE TO CHAPTER 3

In **Chapter 2**, we demonstrated that our vaccine candidate was immunogenic and protected mice from *Clostridioides difficile* challenge. Having achieved the proof-of-concept that the receptor binding domain of toxins A and B expressed by *Salmonella enterica* serovar Typhimurium YS1646 elicits protective responses, we next sought to examine the longevity of these responses in mice. The following chapter describes the antibody and cell-mediated responses 6 months after vaccination. Additionally, mice were challenged 6 months after vaccination, to observe the efficacy of the long-lasting responses.

This chapter was adapted from the following manuscript: Attenuated *Salmonella* Typhimurium vectored vaccine provides long-term protection against lethal *Clostridioides difficile* challenge. **Winter, K.**, *et al.* (prepared for submission to *Vaccines*). This manuscript was prepared as a brief communication, therefore the discussion is telegraphic. A more detailed discussion of this work is presented in **Chapter 5**.

Chapter 3: Attenuated *Salmonella* Typhimurium vectored vaccine provides long-term protection against lethal *Clostridioides difficile* challenge

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*Manuscript prepared for submission to *Vaccine* as a brief communication

3.1 ABSTRACT

Recently, several vaccine candidates for *Clostridioides difficile* based on traditional approaches have either stalled or failed outright during clinical development. We have repurposed attenuated strains of *Salmonella* Typhimurium to express the receptor binding domains of either toxins A or B and demonstrated short-term efficacy using either an oral only or a multimodal approach. In this work, we determined the protective efficacy of our vaccine candidates, 6 months after vaccination in mice. Mice were given 3 oral doses of antigen-expressing *Salmonella* Typhimurium, with or without the corresponding recombinant antigen delivered intramuscularly. Intramuscular immunization maintained high levels of antigen-specific IgG. Oral immunization elicited high intestinal IgA titers that were maintained for 6 months. Administration of antigen-expressing YS1646 strains induced toxin-specific responses in splenocytes that persisted to 6 months. All vaccine strategies protected mice from lethal challenge 6 months after vaccination. The novel *S.* Typhimurium vaccine candidates elicited long-lasting protective immunity in mice.

3.2 KEYWORDS

Clostridioides difficile, *Salmonella* Typhimurium, vaccination, long-term immunity, humoral immunity, cell-mediated immunity

3.3 INTRODUCTION

Clostridioides difficile infection (CDI) is one of the most important and expensive nosocomial infections (1). In 2015, CDI cases cost an estimated 6 billion USD annually in the US (2). In addition to increasing rates of community-acquired CDI, the populations at highest risk, (eg: the elderly) are steadily increasing in most resource-rich countries (1, 3). Despite the role of antibiotics and a disrupted gut microbiome in predisposing to CDI, other treatment options still rely primarily on further antibiotics. Recurrences of CDI occur in 25-30% after recovery from the initial episode. There is clearly a need for alternate approaches, including the development of effective vaccines (3, 4). Several candidate vaccines that appeared promising in pre-clinical testing advanced to Phase 2 and Phase 3 trials where they have either stalled (Valneva) or failed (Sanofi/Pfizer) (5). The design of candidates has been similar; all target *C. difficile* toxins A and B using multiple intramuscular (IM) doses, over several months. Our lab has repurposed an attenuated strain of *Salmonella enterica* serovar Typhimurium YS1646 to express the receptor binding domains (RBD) of toxins A and B of *C. difficile* (6). YS1646 was originally designed as a non-specific cancer therapeutic and was proven to be safe after intravenous injection in patients with advanced cancer in a phase I clinical trial (7). Using a multi-modal vaccination strategy (3 oral (PO) doses in combination with a single IM dose of recombinant antigen), we have recently demonstrated short-term protection from a lethal challenge in a mouse model. Longer-term memory responses generated by *Salmonella*-vectored vaccines have not been well studied. In this work we examined the immune responses elicited by our vaccine candidates and their ability to provide protection up to 6 months after vaccination.

3.4 RESULTS AND DISCUSSION

To examine the longevity of the responses elicited by vaccination, mice were vaccinated and followed for 6 months. We used a multimodal strategy that we had previously established elicits high IgG titers in the serum and a slight increase in IgA titers in the intestine (6). Mice were vaccinated IM with recombinant antigen (rrbdA and/or rrbdB) on day 0 and given 3 PO doses on days 0, 2 and 4 of antigen expressing YS1646 (pagC_SspH1_rbdA and/or SspH2_SspH2_rbdB). Mice that received an IM dose of recombinant protein generated high antigen-specific IgG titers (Fig 3.1A). These titers were maintained throughout 6 months. We observed some cross reactivity in mice vaccinated against TcdA, as they had significantly higher

rrbdB-specific IgG titers at 6 months compared to the PBS control. Mice vaccinated IM+PO against both toxins had significantly increased IgA titers against both toxins 6 months after vaccination (Fig 3.1B). PO vaccination against both toxins elicited a significant increase in rrbdB-specific IgA titers.

While the correlates of protection against *C. difficile* are still not fully established, CDI is primarily a toxin-mediated disease. High anti-toxin IgG and IgA titers are both correlated with decreased severity of illness or reduced reoccurrence rates. Th2-type responses that support antibody production and isotype switching in B cells to IgA, such as IL-5 secretion, are likely to be beneficial in a vaccine-induced response (8-12). T_H2–type CD4+ T cells are thought to play a major role in maintaining long-term humoral response capability (13). Type 1 innate lymphoid cells producing IFNγ and IL-17 may help coordinate early resistance responses to *C. difficile* and recently, it has been suggested that TcdB responsive, IL-17 producing $CD4^+$ cells may correlate better with protection from disease state than humoral responses (14-16). These data suggest that an ideal vaccine candidate for *C. difficile* should elicit T_H1, T_H2, and T_H17 responses.

To investigate the long-term cell mediated immunity generated by our vaccines, we stimulated splenocytes with recombinant rbd antigens 6 months after vaccination and examined the secretion of 16 cytokines and chemokines over 72h. Vaccinated mice had a different pattern of response compared to the PBS control (Fig 3.2A). The response to rrbdA stimulation was reduced in the IM only group, with significant decreases in IL-5 and IL-17 secretion compared to unvaccinated mice (Fig 3.2B). Mice vaccinated IM+PO against rbdA with or without rbdB, had stronger T_H1 responses, as well as a trend in increased IL-4, IL-17, and MCP-1 production. Mice vaccinated PO only against both rbdA and rbdB had a response much closer to the PBS control, with a trend toward increases in IL-1β, IFNγ, IL-3 and MCP-1 production.

After stimulation with rrbdB, mice vaccinated IM against rbdA and rbdB had decreased cytokine and chemokine secretion compared to the PBS control, with a significant decrease in IL-5 production (Fig 3.2). Mice vaccinated either PO only or IM+PO against rbdB (with or without rbdA) generally had similar responses (Fig 3.2A). These groups had slightly increased IL-1α, IL-6, and IFNγ secretion compared to unvaccinated mice. PO only vaccination increased

IL-10, IL-3, and GM-CSF secretion in response to rrbdB stimulation. IM+PO vaccination increased MCP-1 secretion. There were no significant differences in IL-17 secretion between the groups, but there was a trend towards increased secretion by mice vaccinated IM+PO (Fig 3.2B). Although few of the differences achieved statistical significance, mice vaccinated PO had increased responses in the spleen for almost all of the cytokines/chemokines assayed compared to mice vaccinated IM only. Multimodal vaccination generally elicited the greatest cytokine/chemokine responses and were overall more robust following rbdA than rbdB vaccination.

To establish the long-term protective efficacy of these candidate vaccines, we challenged mice with a lethal dose of *C. difficile* 6 months after vaccination. All vaccination strategies significantly protected mice from challenge (83-100%) compared to the PBS control mice that had only 33% survival (Fig 3.3A). All vaccination strategies also reduced the clinical scores of mice during infection (Fig 3.3B). None of the mice in the positive control group that received rrbdA + rrbdB IM with PO delivery of a YS1646 containing an empty plasmid, pQE_null, experienced significant illness and only a small number mice vaccinated IM+PO against rbdA and/or rbdB had high clinical scores (≥10). The IM+PO vaccinated mice with high clinical scores only had them for a short period of time (under 24h). A quarter of PO only vaccinated mice experienced high clinical scores for an extended period. These data correspond well with the pattern of protection we observed 5 weeks after immunization using these same vaccination strategies (6). Based on the clinical scores, it appears that IM vaccination is sufficient for protection 6 months after vaccination in our mouse model. However, the essentially identical strategy of IM immunization has been tested in two phase 3 clinical trials with similar toxin-targeting candidates and both failed to provide substantial protection in humans. Sanofi's *Cdiffense*™ phase 3 was terminated, when the efficacy was determined to be -5.2% [95% CI, -104.1 to 43.5] at the interim analysis (NTC01887912) (17). More recently, Pfizer's CLOVER phase 3 ended with a vaccine efficacy estimate of only 31% (NTC03090191) (5). These vaccine candidates had been demonstrated to elicit high serum IgG titres capable of neutralizing *C. difficile* toxins (18, 19). The failure of these IM delivered, toxin-targeting vaccines to provide adequate protection in humans highlights the need for novel vaccine strategies.

3.5 CONCLUSION

This study examined long-term immune responses elicited by vaccination with a multimodal or PO only *S.* Typhimurium-vectored vaccines. The follow-up period of six months used in this study represents a sixth to a quarter of the average lifespan of a mouse (20). While all our findings may not be directly translatable to humans, they allow us to hypothesize that our novel vaccine and administration schedule may have the capacity to generate long-lived immune responses in people. While PO vaccination may be sufficient to protect humans, multimodal vaccine administration would be simple to implement in a clinic. The IM and first PO dose could be administered in the clinic and the remaining PO doses taken at home. This study also contributes to our limited knowledge on the long-term responses to vaccination with an attenuated *S.* Typhimurium vector. In parallel with work reported here, we have developed YS1646 strains with chromosomally-integrated expression of our targeted antigens. We plan to move these candidate vaccines forward into clinical development either alone (eg: strains expressing rbdB) or combined (rbdB + rbdA).

3.6 METHODS

3.6.1 Bacterial Strains

Salmonella enterica Typhimurium YS1646 (Δ*msbB2* Δ*purI* Δ*Suwwan xyl* negative; ATCC 202165; ATCC, Manassas, VA) was obtained from Cedarlane Labs (Burlington, ON, Canada). Plasmids were designed and electroporated into YS1646 in a previous study (6). *Escherichia coli* BL21 (DE3) cells (Novagen, MilliporeSigma, Burlington, MA) were used for expression and purification of the recombinant $TcdA_{1820\text{-}2710}$ (rbdA) and $TcdB_{1821\text{-}2366}$ (rbdB) (6). *Clostridioides difficile* strain VPI 10463 (ATCC 43255) was obtained from Cedarlane Labs.

3.6.2 Mice

Six- to eight-week-old female C57BL/6J mice were obtained from Charles River Laboratories (Montreal, QC, Canada) and were kept under pathogen-free conditions in the Animal Resource Division at the McGill University Health Center Research Institute (RI-MUHC). Animal procedures were approved by the Animal Care Committee of McGill University and performed in accordance with the guidelines of the Canadian Council on Animal Care.

3.6.2.1 Vaccination

Mice were vaccinated as previously described (6). Briefly, mice were gavaged with $1x10^9$ cfu of the YS1646 strains (days 0, 2, and 4). Intramuscular injections contained 10 μg of recombinant protein and 250 μg of aluminum hydroxide gel (Alhydrogel; Brenntag BioSector A/S, Denmark).

3.6.2.2 Blood and Intestine Sampling

Baseline and monthly serum samples were collected from the lateral saphenous vein prior to all other study procedures using Microtainer serum separator tubes (Sarstedt, Germany). Serum samples were also collected from the mice at the end of the study by cardiac puncture after isoflurane-CO_2 euthanasia. At study termination, 10 cm of the small intestine, starting at the stomach, was collected (6). The tissue was stored in a protease inhibitor cocktail (P8340; Sigma-Aldrich). For post challenge data, samples were collected from survivors 3 weeks after infection.

3.6.2.3 Spleen Collection

Spleens were excised and collected from mice 6 months after vaccination in Hank's balanced salt solution (HBSS) with 50 μg/ml of gentamycin (Wisent) and kept on ice. Spleens were crushed and splenocytes were passed through a 70 μm cell strainer (BD Biosciences). Cells were treated with ammonium-chloride-potassium cell lysis buffer for 3 min. They were then washed twice with HBSS. Cells were resuspended in Gibco Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1 mM penicillin/streptomycin (Wisent), 10% Fetal Bovine Serum (FBS) and β-mercaptoethanol (Sigma-Aldrich).

3.6.2.4 Clostridioides difficile Challenge

C. difficile challenge experiments were performed as described previously (6, 21, 22). Mice were challenged with $1.04x10^7$ and $1.38x10^7$ cfu/mouse of freshly cultured *C. difficile*. Mice were then monitored and scored 1 to 3 times daily for weight loss, activity, posture, coat quality, diarrhea, and eye/nose symptoms (21). Mice with a score of 14/20 or above and/or with

a ≥20% weight loss were considered at a humane endpoint and were euthanized. Survivors were followed and euthanized approximately 3 weeks after infection.

3.6.3 ELISA

ELISAs were performed as previously described (6).

3.6.4 Cytokine Quantification (Quansys)

Splenocytes were plated at $3x10^6$ cells/well and were stimulated with 2 μg/ml of rrbdA or rrbdB for 72h. Supernatant was collected and the concentrations of 16 cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1/CCL2, IFNγ, TNFα, MIP-1α/CCL3, GM-CSF and RANTES/CCL5) were determined using Q-Plex™ Mouse Cytokine - Screen (16-plex) multiplex ELISA following the manufacturer's guidelines (Quansys Biosciences, Utah). Samples were run in singlet.

3.6.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 9) software. For analysis of antibody titers and cytokine concentrations, a one-way nonparametric Kruskal-Wallis analysis of variance was performed with Dunn's multiple-comparison analysis for comparison of all groups to the PBS control. Statistical significance was considered to have been achieved when P was ≤0.05. Data are presented as the means ± standard deviations (SD) or the median ± 95% confidence interval. For analysis of survival, the log-rank (Mantel-Cox) test was used to compare all groups to the PBS control group. The Bonferroni method was used to correct for multiple comparisons.

3.7 DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3.8 ACKNOWLEDGEMENTS

We would like to thank Annie Beauchamp for assistance with all animal work carried out in this study. Thanks go to Dr. Li Xing for his work on the initial development of the plasmids.

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3.9 AUTHOR INFORMATION

All authors contributed to the study and experimental design. K.W. performed all experiments. K.W. and B.J.W. performed the analysis of the data and prepared the manuscript.

3.10 COMPETING INTERESTS

K.W. and B.J.W. are inventors on a patent for YS1646 as a vaccine against *Clostridioides difficile* held by Aviex Technologies LLC.

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3.12 FIGURES AND LEGENDS

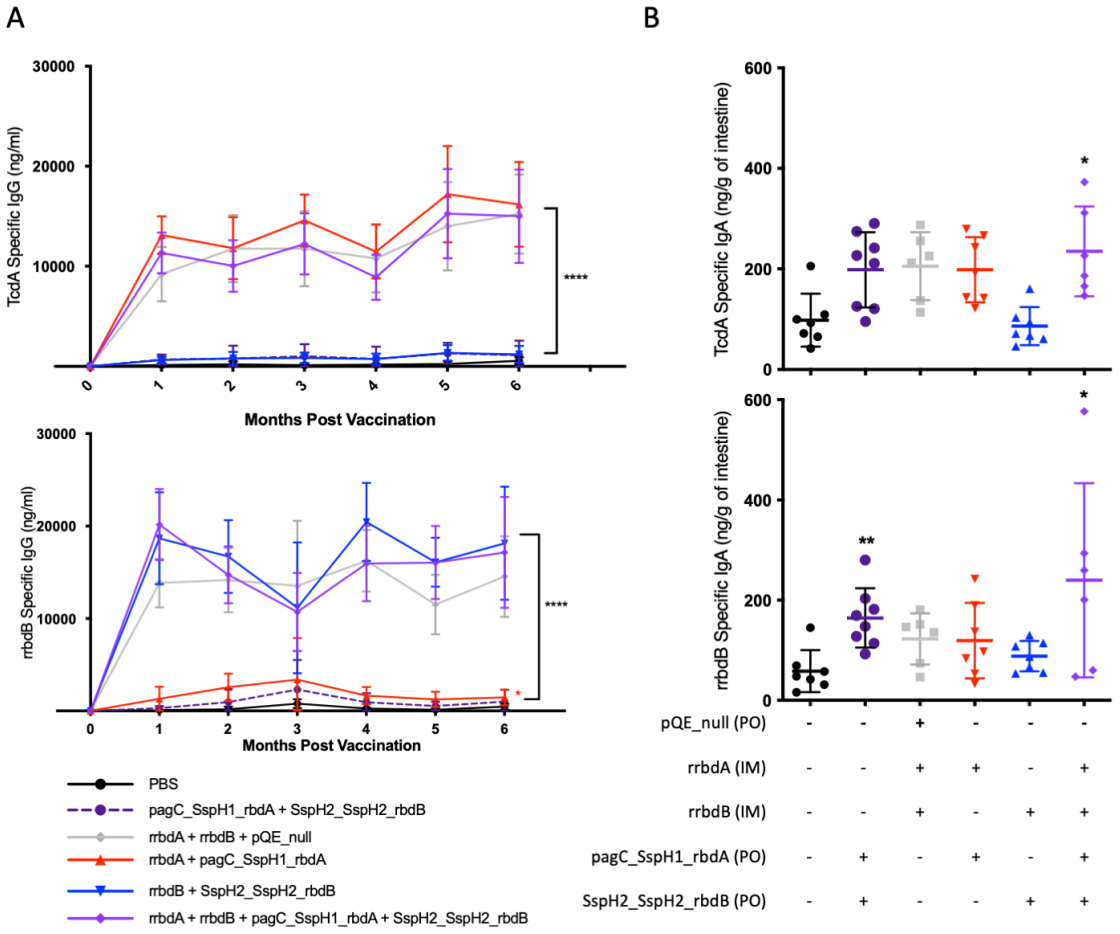

Figure 3.1 Serum IgG and Intestinal IgA titers elicited by vaccination are maintained up to 6 months after vaccination. Mice were immunised with 10 µg of recombinant antigen (rrbdA and/or rrbdB) intramuscularly on day 0 and orally with three doses of $1x10^9$ cfu of antigen expressing YS1646 (pagC_SspH1_rbdA and/or SspH2_SspH2_rbdB) on days 0, 2 and 4. A) Serum was collected monthly and TcdA-specific IgG titers (top panel) and rrbdB-specific IgG titers (bottom panel) were determined by ELISA (n=17-34, 2 repeats). B) 6 months after vaccination, TcdA-specific intestinal IgA titers (top panel) and rrbdB-specific intestinal IgA titers (bottom panel) were determined by ELISA (n=6-8, 2 repeats). All data is shown as the mean ± standard deviation. The Kruskal-Wallis test and Dunn's multiple comparison test were used to compare all groups to the PBS group at 6 months. Coloured stars correspond with the group. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

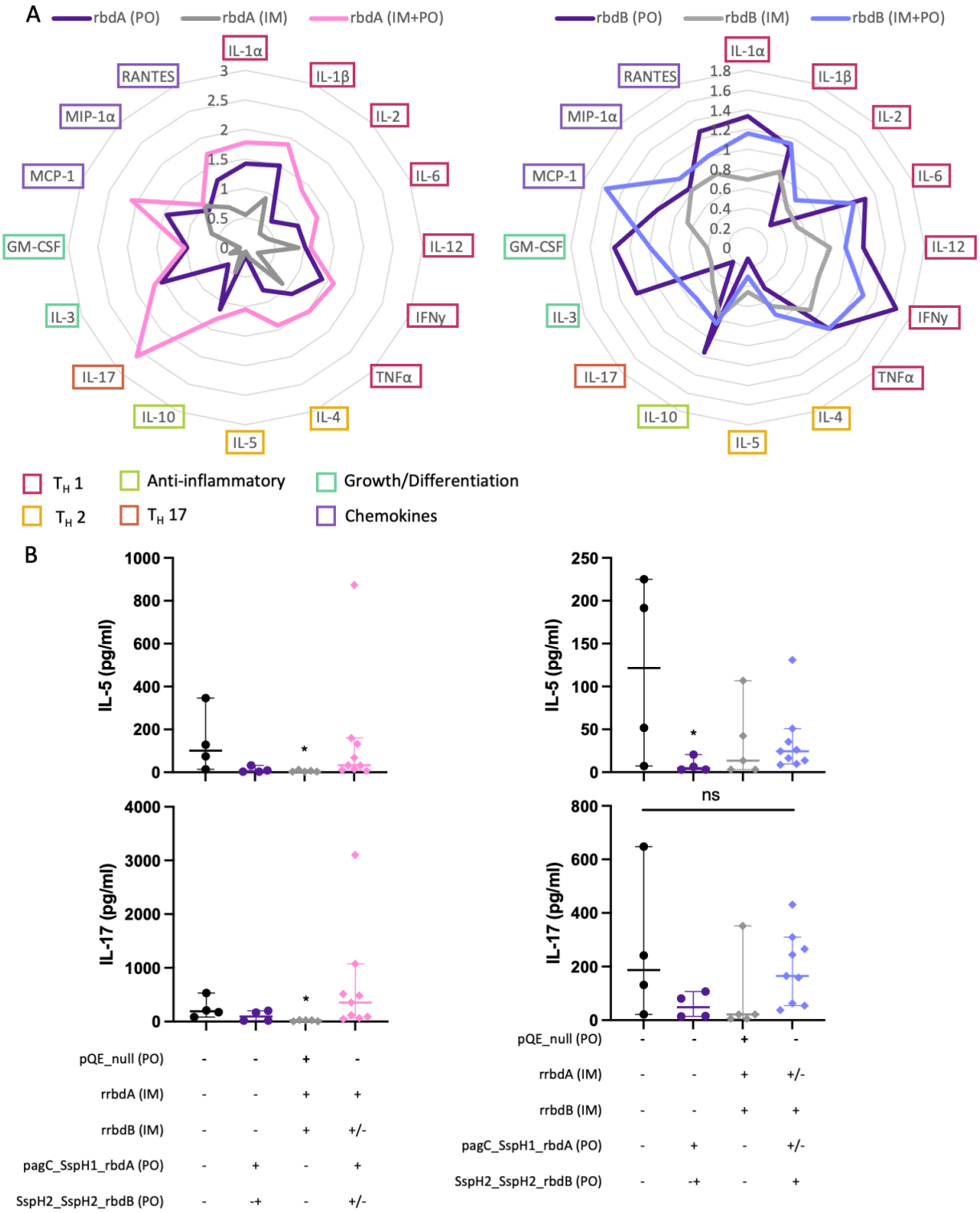
A
rbdA (PO)
rbdA (IM)
rbdA (IM+PO)
rbdB (PO)
rbdB (IM)
rbdB (IM+PO)
IL-1α
IL-1β
IL-2
IL-6
IL-12
IFNγ
TNFα
IL-4
IL-5
IL-10
IL-17
IL-3
GM-CSF
MCP-1
MIP-1α
RANTES
T_H 1
T_H 2
T_H 17
Anti-inflammatory
Chemokines
Growth/Differentiation
B
IL-5 (pg/ml)
IL-17 (pg/ml)
ns
pQE_null (PO)
rrbdA (IM)
rrbdB (IM)
pagC_SspH1_rbdA (PO)
SspH2_SspH2_rbdB (PO)

Figure 3.2 The pattern of response in splenocytes 6 months after vaccination is altered. Mice were immunised with 10 μg of recombinant antigen (rrbdA and/or rrbdB) intramuscularly on day 0 and orally with three doses of $1x10^9$ cfu of antigen expressing YS1646 (pagC_SspH1_rbdA and/or SspH2_SspH2_rbdB) on days 0, 2 and 4. 6 months after vaccination splenocytes were collected and stimulated with recombinant antigen (rrbdA or rrbdB) for 72h. Secreted cytokines and chemokines were analysed by ELISA. A) The fold change of the mean of secreted cytokines and chemokines from the PBS control is shown between groups that received antigen PO, IM or IM+PO. Mice that received either one or both antigens IM+PO were combined to increase the n. Cells stimulated with rrbdA are shown on the right, and those stimulated with rrbdB are on the left (n=4-9, 1 repeat). B) IL-5 (top panels) and IL-17 (bottom panels) secretion after rrbdA stimulation (right panels) and rrbdB stimulation (left panels) is shown (n=4-9, 1 repeat). Data is shown as the median ± 95% confidence interval. The Kruskal-Wallis test and Dunn's multiple comparison test were used to compare all groups to the PBS group. *, $P < 0.05$.

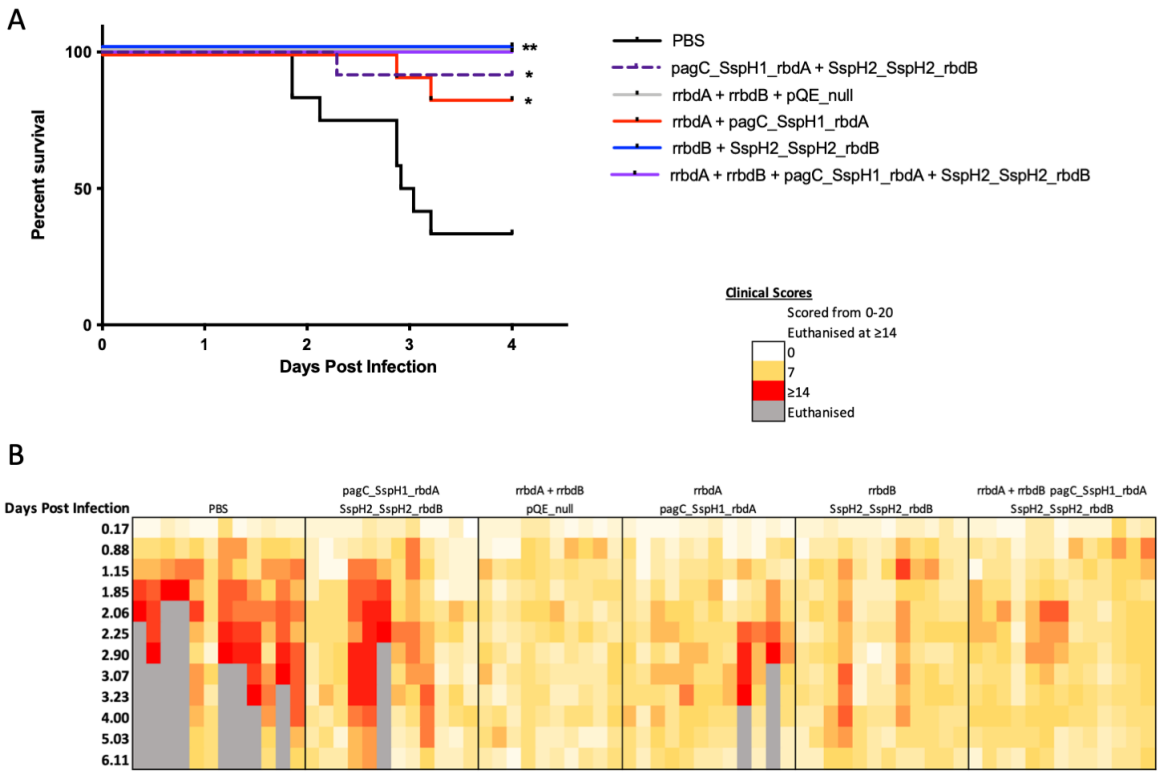

Figure 3.3 Multimodal and oral vaccination protects mice from lethal *C. difficile* challenge 6 months after vaccination. Mice were immunised with 10 µg of recombinant antigen (rrbdA and/or rrbdB) intramuscularly on day 0 and orally (PO) with three doses of $1x10^9$ cfu of antigen expressing YS1646 (pagC_SspH1_rbdA and/or SspH2_SspH2_rbdB) on days 0, 2 and 4. 6 months after vaccination mice were challenged PO with $1.04x10^7$ and $1.38x10^7$ cfu of freshly cultured *C. difficile*. Mice were clinically scored 1 to 3 times daily by an observer blind to the treatment. A score of 14/20 or higher or a weight loss of greater than 20% of the starting body weight was considered the clinical endpoint and mice were euthanised. Survival (A) and clinical scores (B) are shown (n=10-13, 2 repeats). The log-rank (Mantel-Cox) test was used to compare all groups to the PBS control group. Correction of the *P* value for multiple comparisons was done using the Bonferroni method. *, $P < 0.01$; **, $P < 0.002$.

PREFACE TO CHAPTER 4

In **Chapter 3**, we demonstrated that the antibody responses elicited by our vaccine candidate were maintained for 6 months. Oral vaccination with antigen-expressing YS1646 elicited increased responses to antigen stimulation 6 months after vaccination in splenocytes. These responses were protective against *Clostridioides difficile* challenge. Taken together with the data from **Chapter 2**, we were confident that our vaccine candidate has merit and could be taken further into clinical testing. However, the presence of both an antibiotic resistance cassette and the antigen sequence on a mobile genetic element, meant that our vaccine candidate was not suitable for human use. In the following chapter, we sought to integrate our antigen sequence into the *Salmonella enterica* serovar Typhimurium YS1646 genome. After this was accomplished, we examined the immune response elicited and the protective efficacy of our chromosomally integrated vaccine candidates.

This chapter was adapted from the following manuscript: Vaccination with a chromosomally integrated Salmonella Typhimurium vector protects mice from lethal Clostridioides difficile challenge. **Winter K.**, *et al*. (prepared for submission to npj vaccines)

Chapter 4: Vaccination with a chromosomally integrated *Salmonella* Typhimurium vector protects mice from lethal *Clostridioides difficile* challenge

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*Manuscript prepared for submission to *npj vaccines*

4.1 ABSTRACT

Developing a vaccine against *Clostridioides difficile* is key strategy to help protect the elderly. We have repurposed an attenuated strain of *Salmonella* Typhimurium (YS1646) to deliver the receptor binding domains of *C. difficile* Toxin A (TcdA) and Toxin B (TcdB). In this study, YS1646 candidates with TcdA and TcdB expression cassettes stably-integrated into the bacterial chromosome were generated and used in a short-course multimodal vaccination strategy that combined oral delivery (PO) of the YS1646 candidate(s) on days 0, 2 and 4 and intramuscular delivery (IM) of recombinant antigen(s) on day 0. PO and multimodal vaccination against TcdA completely protected mice from a lethal *C. difficile* challenge. Multimodal vaccination elicited high IgG titers, and IL-5 secretion and an increase in IgA^+ plasma cells at the mesenteric lymph node. With the established safety profile of YS1646 we hope to move this vaccine candidate forward into a Phase I clinical trial.

4.2 INTRODUCTION

Clostridioides difficile infection (CDI) is one of the most important nosocomial infections. In the United States in 2017, CDI led to 223 900 hospitalized cases and 12 800 deaths, with an estimated cost of close to $6 billion (1, 2). In part due to better decontamination protocols and transmission control, hospital-acquired CDI rates have been dropping since 2009 (3, 4). However, two thirds of cases occur in patients over the age of 65, and the number of elderly in the US is expected to double by 2050 (5-7). The highest risk for CDI is during administration of antibiotics and during the first month after cessation (6). Paradoxically in many respects, current treatment strategies for CDI are based primarily on prescribing additional antibiotics, and up to 35% of the patients who initially recover experience disease recurrence within the following 3-6 months (8). While fecal microbiome therapy is a highly publicised second-line treatment option, it is cumbersome, not widely available and has its own spectrum of complications (6). Prevention, in the form of transmission control, is a key strategy in reducing the CDI disease burden but vaccination to either prevent disease or to reduce the rate of recurrence would be another powerful tool.

CDI is a toxin mediated disease; most strains produce two main cytotoxins: Toxin A (TcdA) and Toxin B (TcdB). Both toxins irreversibly glycosylate Rho GTPases in intestinal epithelial cell cytosol leading to the disruption of the cytoskeleton and tight junctions, loss of stress fibers and an overall loss of intestinal barrier function (9-14). The toxins are immunogenic and anti-toxin antibodies can have strong neutralization activity *in vitro* (15). Currently, the three vaccines that have reached the stage of Phase 2/3 clinical trials have targeted these toxins using intramuscular (IM) vaccination with adjuvants to generate a systemic immune response (16-19). Two of these candidate vaccines have failed to meet their primary endpoints in Phase III and further development of one was formally abandoned (20, 21). These high-profile failures suggest that novel strategies are needed in the design of vaccines for *C. difficile*. Since *C. difficile*, is an extracellular and non-invasive pathogen of the gut, we reasoned that a vaccine capable of generating both systemic and mucosal immunity might be more successful in targeting the *C. difficile* toxins in the gut lumen.

Salmonella enterica has been investigated as potential live-attenuated vaccine vector for decades (22). The success of *S. enterica* serovar Typhi (*S.* Typhi) Ty21a as a live-attenuated vaccine against typhoid in the 1980s clearly demonstrated the ability of attenuated *Salmonella* to induce a protective response (23). While wild-type *S.* Typhi causes a systemic infection, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is restricted to the gastrointestinal tract in humans (24). Like all Salmonella species, *S.* Typhimurium uses its type 3 secretion systems (T3SS) to maintain an intracellular life cycle in host intestinal epithelial cells and macrophages. Early effector proteins secreted by the *Salmonella* pathogenicity island I (SPI-I) T3SS promote entry into host cells. Once inside the cell and relatively protected inside a *Salmonella* containing vacuole (SCV), proteins secreted by a second T3SS, SPI-II, are used to maintain the SCV. The SPI-I and SPI-II T3SSs can be co-opted in vaccine design to deliver heterologous proteins in the gut and adjacent immune tissues by attenuated strains of *S.* Typhimurium.

Our group is repurposing a strain of *S.* Typhimurium, YS1646, that was originally designed as a possible cancer therapeutic in the late 1980s. After extensive testing in multiple animal models, it was used in a large phase I clinical trial in subjects with advanced cancer (25). Although it failed as an anti-cancer 'drug', this research demonstrated that YS1646 was safe even when injected at doses up to 10^8 intravenously (IV). In early work using a plasmid-based antigen expression system, we developed two main vaccine candidates that expressed a portion of the receptor binding domains (rbd) of TcdA and TcdB (26). When delivered in a multimodal vaccination strategy (ie: oral dosing combined with recombinant antigen delivered intramuscularly (IM)) these vaccine candidates were able to protect mice from lethal challenge with *Clostridioides difficile*.

Since these YS1646 strains contain an antibiotic resistance gene on a mobile genetic element, in this work we sought to produce suitable vaccine candidates by stably integrating the genes of our antigens into the bacterial genome at the *att*Tn*7* site on the bacterial chromosome. After screening multiple candidates with integrated *tcdA* or *tcdB* gene domains fused to sequences encoding a type 3 secretion signal and testing expression from different promoters, we chose two strains that had detectable antigen expression in Luria broth (LB) and tested them in a

multimodal vaccination strategy in mice. We observed that IM vaccination elicited high IgG titers, with multimodal vaccination increasing the avidity of TcdB-specific antibodies. Oral (PO) and multimodal vaccination against TcdA completely protected mice from *C. difficile* challenge.

4.3 RESULTS

4.3.1 *S.* Typhimurium YS1646 expresses recombinant proteins from chromosomally-integrated genes

To generate stably expressing recombinant antigens from genes integrated into the *Salmonella* Typhimurium YS1646 chromosome, 6 different sets of promoter, secretion signal and antigen sequence combinations were designed and integrated into the *att*Tn*7* site (Table 4.1). Combinations were selected based on previous research using plasmid-based antigen expression (26). The promoters used were either constitutively active (*pfrr*) or induced when *Salmonella* is enters host cells (*ppagC* and *pSspH2*). Two secretory signals were used that drive secretion at different times during bacterial invasion and intracellular residence were used. SspH2 is specific to the SPI-II T3SS, while SspH1 can be secreted by both SPI-I and SPI-II T3SSs. The same regions encoding of the receptor binding domain of TcdA (rbdA) or TcdB (rbdB) used in our plasmid-based vaccines were used as the antigens (26). All primers used in the study are listed in supplemental materials. The sequence for insertion was first placed in the pGp-Tn*7*-Cm plasmid, a mobilizable, pir-dependent suicide vector (Fig. 4.1A). Through bacterial conjugation, the pGp-Tn*7*-Cm derived plasmids were introduced to YS1646 cells containing the Tn*7* transposase vector on a temperature sensitive plasmid. Chromosomal integration occurred at the *att*Tn*7* site, which follows the constitutively active *glmS* gene in *S.* Typhimurium. After successful integration, FRT recombination was used to remove the chloramphenicol resistance cassette, generating YS1646 containing the desired recombinant sequences of interest within the bacterial chromosome in the absence of any antibiotic-resistance gene (Fig. 4.1B).

In vitro antigen expression in the vaccine candidates was evaluated by western blotting. Two candidates (ie: frr_SspH1_rbdA and pagC_SspH1_rbdB) expressed detectable levels of the target antigens following growth *in vitro* (Fig. 4.1C). The increased size of the *Salmonella*-expressed antigens is consistent with the secretory signals that are not present in the positive controls. By contrast, the four other strains generated did not have detectable amounts of antigen

following culture. None of the strains had detectable antigen expression 1 h and 24 h after infection of murine macrophages (data not shown). To ensure the fitness of our vaccine strains post-integration, we assessed growth kinetics in LB over 24h. Only one of the strains (ie: frr_SspH1_rbdA) had a slower growth rate after chromosomal integration, compared to the parent strain YS1646 (~80%) (Fig. 4.1D). Because of their ability to drive detectable levels of recombinant antigen expression *in vitro*, the frr_SspH1_rbdA (YS1646::rbdA) and pagC_SspH1_rbdB (YS1646::rbdB) strains were retained and further used for immunogenicity and efficacy testing in mice.

4.3.2 YS1646::rbdA and YS1646::rbdB elicit high IgG titers when delivered in combination with recombinant rbdA and rbdB delivered intramuscularly

Mice were vaccinated using a multimodal strategy: ie: three doses of the antigen expressing YS1646 orally (PO) on days 0, 2 and 4. On day 0 a dose of recombinant antigen (rrbdA/rrbdB) was also delivered intramuscularly (IM). Delivery of both rrbdA and rrbdB IM with oral delivery of YS1646 not expressing any recombinant antigens served as a positive control, as several groups have shown the IM vaccination using the receptor binding domains of the *C. difficile* toxins is immunogenic and provides a high-level of protection in rodents (26-28). As expected, IM administration of rrbdA, rrbdB or both elicited significant antigen-specific IgG titers in mice 4 weeks after vaccination (Fig. 4.2A, B). There was a trend towards increased IgG titers in groups that received both IM and PO vaccines compared the IM only positive controls, but these differences did not reach statistical significance.

To further characterize the IgG elicited by vaccination we examined the avidity and subtypes of the systemic IgG in our vaccinated groups compared to the positive control. TcdA-specific IgG avidity was significantly increased in the groups vaccinated only against TcdA, whether they received IM only or IM + PO vaccination (Fig. 4.2C). IM + PO vaccination against both toxins significantly increased the rrbdB-specific IgG avidity compared to the positive control and vaccination IM + PO against TcdB (Fig. 4.2D). Mice that received either or both antigens IM generated an IgG1 dominated response, with very low, if any, IgG2c titers (Fig. 4.2E, F). Mice vaccinated against TcdA only, generated some cross reactive IgG1 and IgG2c antibodies directed against rbdB, with a skewing towards IgG2c. While PO vaccination alone

generated IgG titers only in a proportion of the mice, the IgG antibodies were skewed to an IgG2c dominated response (Fig. 4.2E, F).

4.3.3 Vaccination against TcdA using a multimodal strategy, including rbdA expressing YS1646, generates a detectable mucosal response

Since the antigen expressing YS1646 targets the gut mucosa, the mucosal response to vaccination was evaluated in mice vaccinated with YS1646::rbdA PO, rrbdA IM or both. Mesenteric lymph nodes (mLN) and Peyer's patches (PP) were collected 32 days after vaccination and the supernatant from cells stimulated with rrbdA for 72 h were tested for the presence of 16 cytokines and chemokines. IL-1α secretion was significantly increased after IM rrbdA vaccination, with a trend towards increased secretion in the IM + PO vaccinated mice compared to the PBS control (Fig. 4.3A). IL-5 secretion in the mLN was significantly increased after IM + PO vaccination. IL-6, IFNγ, IL-17 and GM-CSF had a non-significant increase in secretion in IM and IM + PO vaccinated mice, compared to the PBS and PO only mice (Supp Fig. 4.1A). In the PPs, PO vaccination increased GM-CSF secretion significantly (Fig. 4.3A). Cells from the PPs of mice vaccinated PO or IM alone also had non-significant increases in IL-6 and IFNγ secretion (Supp Fig. 4.1B). A screening for IgA^+ plasma cells (PCs) in the mLNs and PPs was performed using ELISpot. There was an increase ($P = 0.0979$) in IgA^+ PCs in the mLNs of mice vaccinated IM + PO compared to the PBS control (Fig. 4.3B). There were no significant differences in the PPs of vaccinated mice (data not shown).

4.3.4 Vaccination against both toxins or at high doses against TcdA provides protection to mice from *Clostridioides difficile* challenge

Mice were challenged with *Clostridioides difficile* 5 weeks after vaccination. 33% of PBS inoculated control mice survived the infection. Vaccination PO against TcdA and IM + PO against TcdB did not significantly protect mice from challenge (Fig. 4.4A). Mice that were vaccinated IM or IM + PO against TcdA had higher survival than the PBS inoculated mice although it did not reach statistical significance (80% and 69% respectively). IM vaccination against both toxins provided significant protection (Fig. 4.4B). Mice who received the positive control (rrbdA + rrbdB IM) had 100% survival. Mice who had IM + PO vaccination against both toxins had 94% survival. PO vaccination alone did not protect mice from challenge. In fact, mice

that received PO vaccination against both toxins succumbed to infection earlier, with more severe symptoms than the PBS inoculated mice (Fig. 4.4C). Mice vaccinated against both toxins IM or IM + PO had the least severe symptoms compared to all groups. There appeared to be a pronounced cage effect in some groups. Mice vaccinated against TcdA, either IM or IM + PO in one cage had no severe symptoms but in 1-3 of the other cages most mice experienced severe symptoms.

TcdA is more pathogenic in mice than TcdB (29). Based on this, and the non-significant protection effect we observed in our model with mice vaccinated against TcdA, we investigated the protection from challenge after higher vaccination doses with rbdA. Both the IM dose (3 μg to 10 μg) and the PO dose of YS1646::rbdA ($1x10^8$ cfu/mouse to $1x10^9$ cfu/mouse) were increased. Mice were vaccinated IM + PO and PO only against TcdA. 5 weeks after vaccination, mice were challenged with *C. difficile* and both groups had 100% protection (Fig. 4.4D). Both groups of mice experienced almost no severe symptoms (Fig. 4.4E). Only one mouse who received PO only vaccination, experienced a symptom score of 10/20 at one timepoint.

Challenge elicited TcdA-specific IgG titers in most surviving mice, with mice that received rrbdA IM tending to have higher titers than the PBS control (Supp Fig. 4.2A). No rrbdB-specific IgG antibodies were observed in the PBS inoculated mice after challenge, but mice that received rrbdB IM had increased titers compared to the PBS inoculated group. The rrbdB-specific IgG titers were boosted by challenge in mice vaccinated IM + PO against both toxins. The IgG antibodies in all survivors, except mice that received PO vaccination only, were IgG1 skewed (Supp Fig. 4.2B). Mice that received rrbdA IM had a trend of increased TcdA-specific IgG avidity compared to the PBS inoculated control mice (Supp Fig. 4.2C). There were no significant differences in rrbdB specific IgG avidity in survivors (Supp Fig. 4.2D).

IgA titers in the small intestine of surviving mice were examined 3 weeks after challenge. Mice vaccinated IM and IM + PO against both toxins and IM + PO against TcdA had higher TcdA-specific IgA titers compared to PBS mice (Supp Fig. 4.3). All vaccination strategies, including those targeting TcdA alone, tended to increase rrbdB-specific IgA titers, compared to the surviving PBS inoculated mice.

4.4 DISCUSSION

Our primary goal in this work was to develop stable, chromosomally-integrated antigen-expressing *Salmonella* Typhimurium vaccine candidates for *C. difficile*. Previous work had established proof-of-concept that plasmid-bearing, YS1646 expressing repeated motifs of the C-terminal receptor binding domains of either TcdA or TcdB from recombinant plasmids were capable of protecting mice from *Clostridioides difficile* challenge (26). Due to mobility of these plasmids and the presence of an antibiotic resistance cassette, these initial plasmid-containing strains were obviously not suitable for use in humans. While there are several methods for maintaining plasmids in bacteria without the reliance on antibiotic resistance, we chose stable chromosomal integration (30). This permitted us to generate strains with a consistent number of gene copies: initially only one (31). Although this strategy likely reduced expression of the targeted antigens, we tried to mitigate the risk by designing inserts with a strong promoter (*pfrr* and *ppagC*). Two of the chromosomally-integrated strains had detectable levels of heterologous antigen expression by western blot (frr_SspH1_rbdA and pagC_SspH1_rbdB) and both appeared to tolerate the presence of the foreign sequence well, as they had minimal changes in fitness. These two strains were retained for further studies in mice.

There are several limitations to the mouse models used in this study. The main limitation in our vaccination model, is that wild-type *S.* Typhimurium is a mouse pathogen that causes typhoid-like disease in mice (32). It systemically infects mice, with high bacterial burden in the spleen, liver, and gall bladder (33, 34). However, in healthy humans, *S.* Typhimurium is restricted to the gastrointestinal tract and elicits strong mucosal responses (35, 36). We have observed that YS1646 is capable of colonizing the spleen and liver, for up to 3 weeks after vaccination (unpublished data). The immune response to *S.* Typhimurium is tissue specific, which is why we focused on responses elicited in the mesenteric lymph nodes and Peyer's patches. One of the main limitations in the mouse model of CDI is that mice are more sensitive to TcdA than TcdB, while the opposite is believed to be true in humans (29). Syrian hamsters are a very commonly used model for CDI (37). However, their exquisite sensitivity to CDI does not mimic infection conditions in humans (38). Mice require antibiotic treatment, albeit a very robust course of antibiotics, to become susceptible to infection, better modelling the disease in humans.

The positive control used in this study was IM delivery of one or both recombinant antigens (rrbdA and rrbdB) and oral administration of unmanipulated YS1646. This control generated high IgG titers with a sharply skewed IgG1 profile. As expected based on our work and that of others, this vaccine provided good protection against *C. difficile* challenge (26-28). This positive control strategy is similar to most of the other *C. difficile* vaccine candidates that have entered clinical trials. Although repeated doses of these candidates administered IM with adjuvants over several months generated strong serum IgG titers in humans, the two vaccines that advanced to efficacy trials failed to meet their primary endpoint of protection against primary CDI (16, 17, 19). The field trial of Sanofi's aluminum-adjuvanted, formalin-inactivated whole toxin vaccine (*Cdiffense*^{TM}: NTC01887912) was terminated at the interim analysis, when the vaccine efficacy was determined to be -5.2% [95% CI, -104.1 to 43.5] (20). More recently, Pfizer reported disappointing results for their aluminum hydroxide-adjuvanted, genetically detoxified toxin candidate vaccine (NTC03090191). In this multi-year study of >17,000 subjects, three doses of the vaccine were only 31% effective at preventing primary CDI (21). The failure of these candidates, despite the induction of high systemic IgG titers suggests that the possible correlates of protection for CDI should be re-considered in designing the next generation of vaccines. Recently Cook et al demonstrated that TcdB-specific $CD4^+$ T cells in the blood have a stronger negative correlation than humoral responses with disease severity (39). Fecal TcdA-specific IgA titers have been associated with a lower risk of recurrence (40). In a small study, Johal et al observed a decrease in IgA^+ cells in colonic biopsies of patients with CDI that appeared to correlate with disease severity (41).

We have observed that YS1646 delivered antigen at the intestinal mucosa elicits several different responses to vaccination compared to the IM positive control. Our plasmid-based strains elicited an IgA response in the intestine, that is sustained for 6 months (unpublished data) (26). With our chromosomally integrated strains, we demonstrated that the mLN of mice that received multimodal vaccination had significantly increased IL-5 production after stimulation with rrbdA, which could be linked to the increase in TcdA-specific IgA^+ plasma cells (42). Multimodal vaccination against both toxins significantly increased the avidity of the rrbdB-specific IgG antibodies elicited, and PO vaccination elicited an IgG2c-skewed response. The

differences in expression of the cytokines and chemokines measured at the mLN and PPs after vaccination were subtle and generally driven by increased expression from 1-2 mice (Supp Fig. 4.1). However, the cytokines that had increased secretion are interesting in the context of a response to *C. difficile*. Several of the cytokines that were increased, including IL-1α, IL-6 and IFNγ are linked to Th1 responses. IFNγ producing innate lymphoid cells play a role in early resistance to CDI, suggesting that a vaccine that generates a Th1 response would be helpful in early control of the pathogen (43). GM-CSF has been shown to play a role in neutrophil influx in CDI but does not contribute to pathogen clearance (44). Due to its role in gut inflammation, it is not surprising to see an increase in its production after a *Salmonella* infection (45). Finally, IL-17 may play a role in protection against CDI. Cook et al found that TcdB-specific Th17 $CD4^+$ T cells were associated with decreased risk of recurrence (46). Chen et al recently demonstrated that IL-17 production by γδ T cells is vital in neonatal resistance to CDI (47). While the increase in the production of these cytokines was not significant in our vaccine groups, it is positive that the cytokines that had a small signal, would be helpful in the context of CDI.

IgG1 and IgG2c production gives us an indication about the Th skewing of the immune response generated by our vaccines. IgG1 is produced during a Th2 skewed response, while a Th1 response elicits IgG2c antibodies (48). We observed a dichotomy in the production of IgG1 and IgG2c by vaccinated mice. Most mice that produced high IgG titers, produced high IgG1 titers, and very low IgG2c titers. In mice with very low IgG titers, the antibodies produced were mainly IgG2c. This is expected as aluminum hydroxide gel, the adjuvant in our IM delivered vaccine is Th2 skewing, while intracellular infections, such as *S.* Typhimurium elicit Th1 responses (49). Correlates of protection in CDI were discussed above, but as an extracellular pathogen in the intestine, both Th1 and Th2 responses may be needed to clear the infection. With this in mind, multimodal vaccination may be a vital strategy to elicit a mixed Th1/Th2 response.

While examining this work there are several limitations to consider. Protection with a lower dose vaccination and challenge model was dependent on vaccination against both toxins. These data are unlike the data in our higher dose vaccination and challenge model, which we used also in our previous study to demonstrate multimodal high dose vaccination with plasmid bearing YS1646 against either toxin was protective (26). When we used the higher dose

vaccination and challenge model in this study, vaccination against TcdA alone, either IM + PO or PO only had significant protection. Beyond the higher doses of both vaccines one of the major changes in these two models was a change in the source of C57BL/6J mice that we used. Mice acquired from Charles River Laboratories were used in the high-dose studies and were much more resistant to *C. difficile* challenge, requiring a higher inoculation of *C. difficile* to succumb to infection. We hypothesize this is attributed to a difference in the gut microbiota in mice bred in different facilities (50). We speculate that the gut microbiome in Jackson Laboratories bred mice is more susceptible to the antibiotic cocktail leaving a less competitive environmental niche for *C. difficile* colonization. We only examined the mucosal response in the low-dose model, so it is possible that at the higher dose antigen-expressing YS1646 may be better at eliciting protective responses at the gut mucosa. Additionally, our studies examining the mucosal responses had a low number of mice. Considering most of the trends observed were driven by 1-2 mice, increasing the number of mice used could have helped tease apart more differences between the groups. Interestingly, we did observe some evidence of antigen interference in mice vaccinated against both toxins in this study. TcdA-specific IgG avidity was higher in mice vaccinated against TcdA alone, compared to mice vaccinated against both toxins. While bacterial competition of our vaccine strains could be contributing to this issue, as YS1646::rbdB does grow faster than YS1646::rbdA in LB, the trend is also present in mice vaccinated IM only, suggesting that the difference in response is antigenic in nature. Finally, this study did not examine the T cell responses nor the longevity of responses elicited by vaccination.

The ultimate goal of this program is to develop a novel vaccine candidate against *C. difficile* that is safe and can provide both rapid and durable protection in vulnerable populations. One of the main benefits of repurposing the YS1646 strain is its documented safety profile in mice, pigs, non-human primates, and humans (25, 51, 52). The combination of this living vaccine vector in our multimodality regimen elicits a mucosal response that may be vital for protection from CDI. While the multimodality approach adds complications to the regulatory process (ie. more components for safety testing) and packaging, the application of this strategy would be relatively simple in a clinical practice. In a single visit, both the IM dose and the initial PO dose could be administered, with the remaining PO doses taken at home, as is currently done for the oral *S.* Typhi vaccine (53). Although the PO only strategy was generally not as effective

as combined IM + PO vaccination, it is possible that oral vaccination alone with antigen expressing YS1646 may be sufficient to provide protection in humans. To further complicate initial clinical trials, it is possible that vaccination against TcdB may be sufficient to provide protection. However, the vaccines that have undergone Phase II/III clinical trials have all targeted both TcdA and TcdB, so a multi-antigen strategy may be necessary. As one or more of the YS1646 candidates move forward into clinical trials, we will test both administration strategies.

4.5 METHODS

4.5.1 Bacterial Strains and Growth Conditions

Salmonella enterica Typhimurium YS1646 (Δ*msbB2* Δ*purI* Δ*Suwwan xyl* negative; ATCC 202165; ATCC, Manassas, VA) was obtained from Cedarlane Labs (Burlington, ON, Canada). *Escherichia coli* DH5α (Thermo Fisher Scientific, Eugene, OR) was used to produce recombinant Tn*7* plasmids. *E. coli* MGN-617 was used for conjugation with YS1646 (54). Plasmids were introduced into YS1646 either by conjugation or by electroporation (20 ng of plasmid at 1.8 kV, 200 Ω and 25 μF; ECM 399 Electroporation System, BTX, Holliston, MA, US) Plasmids were introduced into *E. coli* by heat shock. *Salmonella* Typhimurium and *E. coli* were cultured in Luria broth (LB), with the following antibiotics when necessary to maintain plasmids; 100 μg/ml of ampicillin, 50 μg/ml kanamycin, 30 μg/ml chloramphenicol, and if necessary 50 μg/ml diaminopilmelic acid (DAP).

For the growth curve, cultures of wild-type YS1646 and chromosomally integrated constructs were grown overnight at 37°C. The next day, the cultures were diluted 1:100 in LB and plated in quadruplicates (n=4) on a 100-well Bioscreen C honeycomb microplate (Growth Curves USA, Piscataway, NJ, USA). The Bioscreen C plate reader measured the optical density of the cultures at a wavelength of 600 nm every 30 minutes over 24 hours with a 30-second shaking period prior to each reading.

Clostridioides difficile strain VPI 10463 (ATCC 43255) was obtained from Cedarlane Labs and used for challenge experiments. Cells were maintained in meat broth (Sigma-Aldrich,

St. Louis, MO) containing 0.1% (wt/vol) L-cysteine (Sigma-Aldrich) in an anaerobic jar. For colony counts, *C. difficile* containing medium was serially diluted and streaked onto brain heart infusion-supplemented (BHIS) plates (BD Biosciences, Mississauga, ON, Canada) containing 0.1% (wt/vol) L-cysteine. The bacteria were left to grow on plates at 37ºC in an anaerobic jar for 24h.

4.5.2 Chromosomal Integration

The chloramphenicol resistant Tn*7* plasmid backbone was originally developed by Crépin et al (31). The pGP-Tn*7*-Cm plasmid backbone was digested using EcoRI and KpnI, and the promoter secretory signal and antigen sequence was inserted using uni seamless cloning and assembly (pEASY kit, TransGen Biotech, Beijing, China). All primers used in the construction of Tn*7* plasmids are described in Supplemental Table 1. Most promoter, secretory signal and antigen sequences were originally made by Winter et al (26). The *frr* promoter was obtained from YS1646 using PCR. After assembly of the Tn*7* plasmids, they were transformed into DAP^- *E. coli* κ712617.

The temperature sensitive pSTNSK plasmid, designed by Crépin et al, containing the Tn*7* transposases system and a kanamycin cassette was transformed into YS1646 (31). The transformed *E. coli* κ712617 and *S.* Typhimurium were incubated together in 10 mL of LB at 30ºC for 5h. They were then plated on LB agar with kanamycin and chloramphenicol but no additional DAP. Individual colonies of YS1646 were selected, grown at 42ºC to lose the pSTNSK plasmid and PCR was performed to confirm chromosomal integration.

Chromosomally integrated YS1646 was transformed with the temperature-sensitive pCP20 plasmid (55), containing an ampicillin resistance cassette, a chloramphenicol resistance cassette and the recombinase flippase (Flp). The chloramphenicol-resistance gene, *cat*, that was integrated at the *att*Tn*7* site of the YS1646 genome was then removed by Flp-FRT recombination. Successful removal of the chloramphenicol resistance cassette in the genome was confirmed by both determining antibiotic susceptibility to chloramphenicol and by PCR.

4.5.3 Recombinant rbdA and rbdB expression

Protein expression and purification of the recombinant $TcdA_{1820-2710}$ (rbdA) and $TcdB_{1821-2366}$ (rbdB) were accomplished using the pET-28b plasmid (Novagen, Millipore Sigma, Burlington, MA) with an isopropyl-β-D-1-thiogalactopyranoside (IPTG) inducible promoter and kanamycin resistance gene. A 6x His tag and stop codon were added at the 3' end. The expression vector was transformed into *E. coli* BLR(DE3) cells (Novagen, Millipore Sigma) by heat shock. Transformed bacteria were grown in a 37°C shaking incubator with 30 μg/ml of kanamycin (Wisent), until the optical density (absorbance) at 600 nm (OD_{600}) reached 0.5 to 0.6. IPTG (Invitrogen, Carlsbad, CA) was then added to a final concentration of 1mM, and expression was induced overnight. Expression of rrbdA was done at 37ºC, while expression of rrbdB was performed at 30ºC. Cells were pelleted by centrifugation at 3,000xg for 30 min at 4°C. Cells were lysed, and the lysate was collected and purified using a denaturing protocol and Ni-nitrilotriacetic acid (NTA) affinity chromatography (Ni-NTA Superflow; Qiagen, Venlo, Limburg, Netherlands). The eluate was analysed by Coomassie blue staining of polyacrylamide gels and Western blotting using a monoclonal antibody directed against the His tag (Sigma-Aldrich).

4.5.4 Western Blotting

For antigen expression *in vitro*, the transformed YS1646 strains were grown overnight in LB at 37°C in 0% CO_2, centrifuged at 21,130xg for 10 min, resuspended in PBS, and then mixed in with NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) according to the manufacturer's instructions. All samples were heated for 10 min at 70°C and then cooled on ice. Proteins were separated on a 4 to 12% Bis-Tris protein gel (Invitrogen) and transferred to nitrocellulose membranes using a Trans-Blot Turbo RTA mini nitrocellulose transfer kit (Bio-Rad, Hercules, CA). For detection of $TcdA_{5458-8130}$ and $TcdB_{5461-7080}$, the membranes were incubated first with anti-toxin A chicken IgY (1:5,000; Abnova, Taipei, Taiwan) and anti-toxin B chicken IgY (1:10,000; Abnova) antibodies, respectively, followed by goat anti-chicken-IgY IgG conjugated to horseradish peroxidase (1:10,000; Thermo Fisher Scientific). Immunoreactive bands were visualized using the SuperSignal West Pico Plus chemiluminescent substrate (Thermo Fisher Scientific) and autoradiography film (Denville Scientific, Holliston, MA).

4.5.4 Mice

Six- to eight-week-old female C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA) or Charles River Laboratories (Montreal, QC, Canada) and were kept under pathogen-free conditions in the Animal Resource Division at the McGill University Health Center Research Institute (RI-MUHC). All animal procedures were approved by the Animal Care Committee of McGill University and performed in accordance with the guidelines of the Canadian Council on Animal Care.

4.5.4.1 Vaccination

For oral vaccinations, mice were gavaged with $1x10^8$ cfu of the YS1646 strains in 0.2 ml of PBS (days 0, 2, and 4). When both strains were given, $5x10^7$ cfu of each strain was used, for a total of $1x10^8$ cfu of YS1646 given in 0.2 ml of PBS. Intramuscular (IM) injections contained 3 μg of recombinant protein and 250 μg of aluminum hydroxide gel (alum; Alhydrogel; Brenntag BioSector A/S, Frederikssund, Denmark) in 50 μl, which was administered into the gastrocnemius muscle using a 28-gauge needle. For the high-dose study mice were gavaged with $1x10^9$ cfu of the YS1646 strains and IM injections contained 10 μg of recombinant protein.

4.5.4.2 Blood and Intestine Sampling

Baseline and pre-infection serum samples were collected from the lateral saphenous vein prior to all other study procedures using Microtainer serum separator tubes (Sarstedt,Nümbrecht, Germany). Serum samples were also collected from the mice at the end of the study by cardiac puncture after isoflurane-CO_2 euthanasia. Serum separation was performed according to the manufacturer's instructions, and aliquots were stored at -20°C until they were used. At study termination, 10 cm of the small intestine, starting at the stomach, was collected. Intestinal contents were removed, and the tissue was weighed and stored in a protease inhibitor (PI) cocktail (catalog number P8340; Sigma-Aldrich) at a 1:5 (wt/vol) dilution on ice until it was processed. The tissue was homogenized (Homogenizer 150; Fisher Scientific, Ottawa, ON, Canada) and centrifuged at 2,500xg at 4°C for 30 min, and the supernatant was collected. Supernatants were stored at -80°C until they were analyzed by enzyme-linked immunosorbent assay (ELISA). For post challenge data, samples were collected from survivors at 3 weeks after infection.

4.5.4.3 Mesenteric lymph node and Peyer's Patches Sampling

Mesenteric lymph nodes (mLN) were excised and collected from mice 30 and 32 days after vaccination, in Gibco Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1 mM penicillin/streptomycin, 10 mM HEPES, 1X MEM non-essential amino acids, 1 mM sodium pyruvate, 1 mM L-glutamine (all Wisent products) and β-mercaptoethanol (Sigma-Aldrich) (cRPMI) and 2% FBS and kept on ice. All mLNs from individual mice were transferred into 1 mL of digestion buffer (1 mg/mL of Collagenase D (Sigma-Aldrich) and 0.1 mg/mL of DNase I (Sigma-Aldrich) in RPMI 1640 + 2% FBS) and cut open. They were then incubated at 37ºC, shaking at 220 rpm for 40 min. The media and mLNs were passed through a 70 μm cell strainer (BD Biosciences) and washed with cRPMI + 2% FBS 3 times. Cells were resuspended in cRPMI + 10% FBS.

4.5.4.4 Clostridioides difficile Challenge

C. difficile challenge experiments were performed essentially as described previously (56-58). Briefly, mice were pre-adapted to acidic water by adding acetic acid at a concentration of 2.15μl/ml (vol/vol) to their drinking water 1 week prior to antibiotic treatments. At 6 days prior to infection, an antibiotic cocktail that included metronidazole (0.215 μg/ml; Sigma-Aldrich), gentamicin (0.035 μg/ml; Wisent), vancomycin (0.045 μg/ml; Sigma-Aldrich), kanamycin (0.400 μg/ml; Wisent), and colistin (0.042 μg/ml; Sigma-Aldrich) was added to the drinking water. After 3 days, regular water was returned, and at 24 h prior to infection, mice received clindamycin (32 mg/kg of body weight; Sigma-Aldrich) intraperitoneally in 0.2 ml of PBS using a 28-gauge needle. Fresh *C. difficile* cultures were used in our challenge model so that the dose used was estimated on the day of infection based on OD_{600} values and the precise inoculum was calculated 24 h later. This procedure led to the use of different *C. difficile* doses in the two repeat challenge studies performed (1370 or 2500 cfu/mouse). In the high dose study, mice received $1.18x10^7$ cfu/mouse. The challenge dose was delivered by gavage in 0.2 ml of meat broth culture medium. The mice were then monitored and scored 1 to 3 times daily for weight loss, activity, posture, coat quality, diarrhea, and eye/nose symptoms (57). A score of 14/20 or above and/or ≥20% weight loss were considered as a humane endpoint and mice were

euthanized. Any mouse found dead was given a score of 20. Survivors were followed and euthanized approximately 3 weeks after infection.

4.5.5 ELISA

Whole toxin A (List Biologicals, Campbell, CA) or recombinant rbdB was used to coat U-bottom high-binding 96-well ELISA plates (Greiner Bio-One, Frickenhausen, Germany). A standard curve was generated for each plate using mouse IgG antibodies, mouse IgG1 antibodies, mouse IgG2c antibodies or mouse IgA antibodies (Sigma-Aldrich). The plates were coated with 50 µl of toxin A (1.0 µg/ml), rrbdB (0.25 µg/ml), or IgG/IgG1/IgG2c/IgA standards overnight at 4°C in 100 mM bicarbonate/carbonate buffer (pH = 9.5). The wells were washed with PBS 3 times and then blocked with 150 µl of 2% bovine serum albumin (BSA; Sigma-Aldrich) in PBS–Tween 20 (0.05%; blocking buffer; Fisher Scientific) for 1 h at 37°C. Serum samples were heat inactivated at 56°C for 30 min before dilution 1:50 in blocking buffer. Intestinal supernatants were not heat inactivated and were added to the plates neat. All sample dilutions, including dilutions for the standard curve, were assayed in duplicate (50 µl/well). The plates were incubated for 1 h at 37°C and then washed 4 times with PBS. For avidity assays, 100 µl of blocking buffer or 6M Urea were added to samples on the same plate for 15 min. Urea was washed off with PBS 4 times and then plates were blocked with 150 µl of blocking buffer for 1 h at 37ºC. After washing the samples or the blocking buffer off (avidity assay) 75 µl of either horseradish peroxidase (HRP)-conjugated anti-mouse total IgG antibodies (1:20,000 in blocking buffer; Sigma-Aldrich), HRP-conjugated anti-mouse IgG1 antibodies (1:20,000 in blocking buffer; Sigma-Aldrich), HRP-conjugated anti-mouse IgG2c antibodies (1:20,000 in blocking buffer; Sigma-Aldrich) or HRP-conjugated anti-mouse IgA antibodies(1:10,000 in blocking buffer; Sigma-Aldrich) were added. The plates were incubated for 30 min (IgG, IgG1, and IgG2c) or 1 h (IgA) at 37°C. Six washes with PBS were performed before the addition of 100 µl/well of 3,3',5,5'-tetramethylbenzidine (TMB) detection substrate (Millipore, Billerica, MA). Reactions were stopped after 15 min with 50 µl/well of 0.5 M H_2SO_4. The plates were read at 450 nm on an EL800 microplate reader (BioTek Instruments Inc., Winooski, VT). The concentration of antigen-specific antibodies in each well (in nanograms per milliliter) was estimated by extrapolation from the standard curve. Avidity Index was calculated as (Antigen-specific IgG concentration remaining after Urea incubation) / (total IgG concentration) x 100%.

4.5.6 ELISpot

A mouse IgA ELISPOT basic kit from Mabtech (Stockholm, Sweden) was used. Briefly, hydrophobic PVDF membrane ELISPOT plates (Millipore Sigma) were coated with 100 µl/well of the anti-IgA capture antibody overnight at 4ºC. Plates were then blocked with cRPMI for at least 30 min at room temperature. mLN cells in cRPMI were added to the plate at $5x10^5$ cells/well and were incubated for 24 h. After washing, 1 µg/ml of biotinylated TcdA was incubated for 2 h at room temperature. The plate was washed and streptavidin-ALP (1:10,000) was added to each well and left to incubate at room temperature for 1 h. BCIP/NBT-plus substrate (Mabtech) was added and the plate developed for 10 min. Plates were read on a CTL series 3B ImmunoSpot analyzer (Cellular Technology Limited, Ohio) with ImmunoSpot 5.2 analyzer software.

4.5.7 Cytokine Quantification (Quansys)

mLN and PP cells were plated in cRPMI at $2.5x10^6$ cells/well and were stimulated with 2 µg/ml of rrbdA for 72h. Supernatant was collected and stored at -80ºC until further use. The concentrations of 16 cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1/CCL2, IFNγ, TNFα, MIP-1α/CCL3, GM-CSF and RANTES/CCL5) were determined using the Q-Plex™ Mouse Cytokine - Screen (16-plex) multiplex ELISA following the manufacturer's guidelines (Quansys Biosciences, Utah). Samples were run in singlet.

4.5.8 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 9) software. For analysis of antibody titers, a one-way nonparametric Kruskal-Wallis analysis of variance was performed with Dunn's multiple-comparison analysis for comparison of all groups. Statistical significance was considered to have been achieved when P was ≤ 0.05. Data are presented as the means $\pm$ standard deviations (SD) or the median $\pm$ the maximum and minimum. For analysis of survival, the log-rank (Mantel-Cox) test was used to compare all groups to the PBS control group. The Bonferroni method was used to correct for multiple comparisons.

4.6 DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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4.8 AUTHOR INFORMATION

All authors contributed to the study and experimental design, with S.H. and C.M.D. lending their considerable expertise to the bacterial genetics experiments. K.W. and S.H. performed the chromosomal integration. K.W. performed all animal experiments. K.W. and B.J.W. performed the analysis of the data and prepared the manuscript.

4.9 COMPETING INTERESTS

K.W. and B.J.W. are inventors on a patent for YS1646 as a vaccine against *Clostridioides difficile* held by Aviex Technologies LLC. S.H. and C.M.D. have no competing interests to declare.

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4.11 TABLES

Table 4.1 YS1646 strains used in this study

Strain	**Promoter**	**Secretory Signal**	**Antigen**
YS1646			
frr_SspH1_rbdA (YS1646::rbdA)	*frr*	SspH1	$TcdA_{1820-2710}$
pagC_SspH1_rbdA	*pagC*	SspH1	$TcdA_{1820-2710}$
SspH2_SspH2_rbdA	*sspH2*	SspH2	$TcdA_{1820-2710}$
frr_SspH1_rbdB	*frr*	SspH1	$TcdB_{1821-2366}$
pagC_SspH1_rbdB (YS1646::rbdB)	*pagC*	SspH1	$TcdB_{1821-2366}$
SspH2_SspH2_rbdB	*sspH2*	SspH2	$TcdB_{1821-2366}$

4.12 FIGURES AND LEGENDS

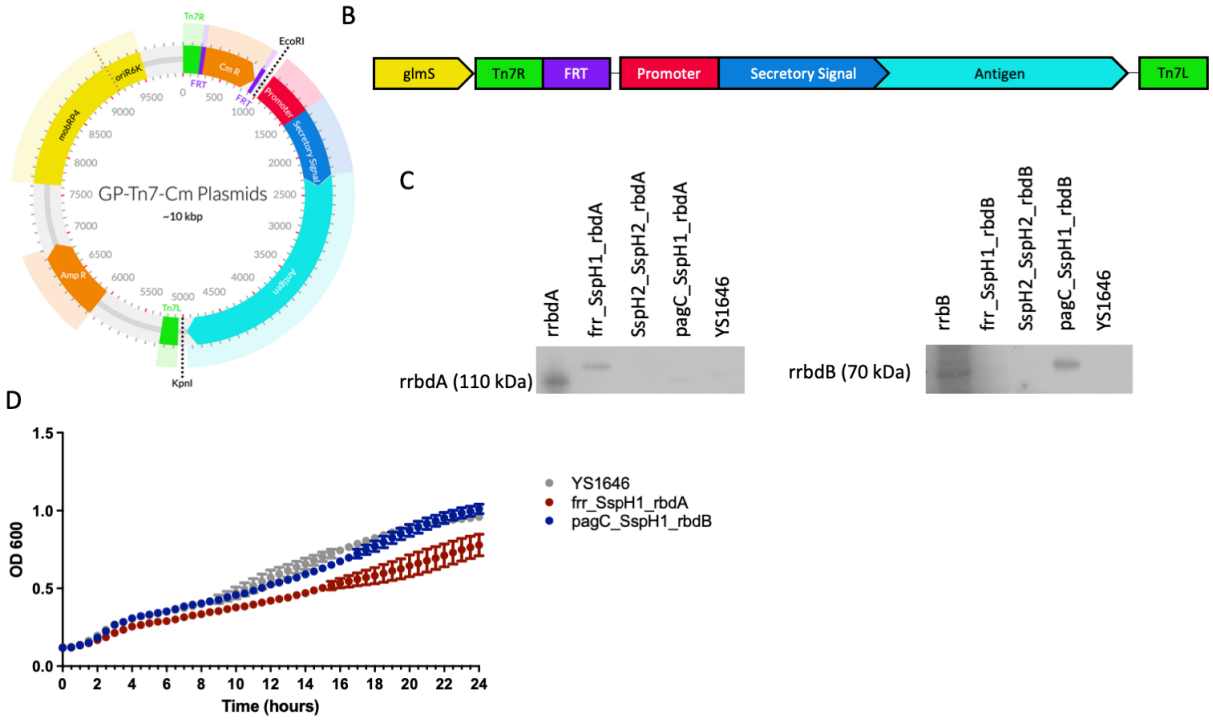
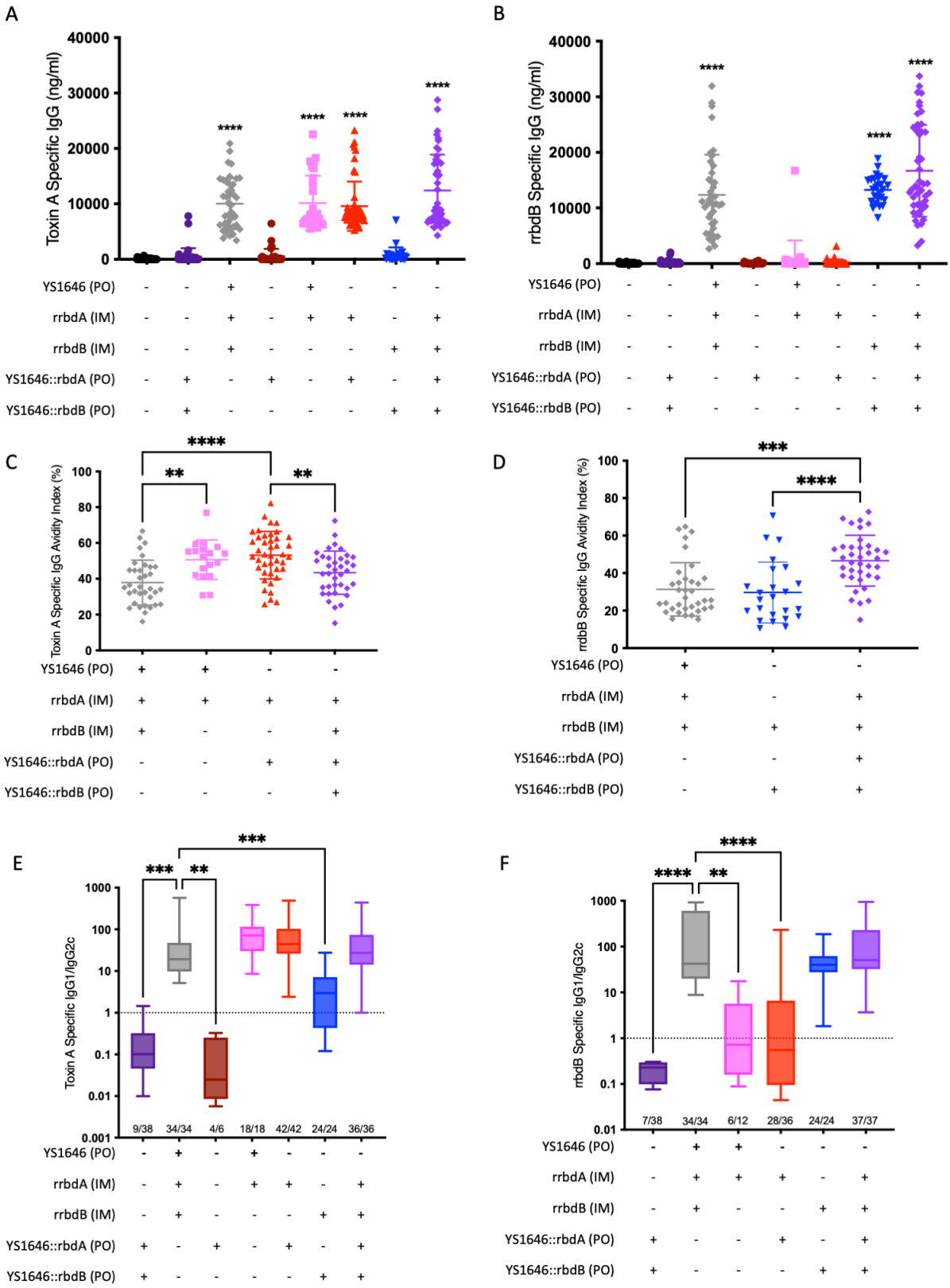


Figure 4.1 *S.* Typhimurium YS1646 expresses recombinant proteins from chromosomally-integrated genes. **A** The promoter, and regions encoding the secretory signal and antigen sequence were inserted into ampicillin- and chloramphenicol-resistant, mobilizable, and *pir*-dependent suicide vectors pGp_Tn*7* plasmids using the EcoRI and KpnI restriction sites and Gibson assembly method. **B** The pGp_Tn*7* plasmids were conjugated into strain YS1646 containing a temperature sensitive plasmid encoding the Tn*7* transposase system. The sequence between the Tn*7* ends containing a chloramphenicol-resistance cassette and either rbdA or rbdB sequences was inserted into the YS1646 genome at the *att*Tn*7* site, adjacent to the glmS gene. After successful chromosomal integration and loss of both plasmids, Flp-FRT mediated recombination was used to remove the chloramphenicol-resistance cassette. **C** The parent strain YS1646, rbdA and rbdB expressing YS1646 were individually grown in LB medium overnight. A western blot was performed with a positive control (rrbdA/rrbdB) produced in *E. coli*. The film was exposed for 2 min. **D** Parent strain YS1646, rbdA and rbdB expressing YS1646 were grown in LB and the OD_{600} was measured every 30 min for 24h (n=4, 1 repeat). Data are presented as the mean and standard deviation (SD) value.


A
Toxin A Specific IgG (ng/ml)
40000
30000
20000
10000
0
****
YS1646 (PO) - - + - + - - -
rrbdA (IM) - - + - + + - +
rrbdB (IM) - - + - - - + +
YS1646::rbdA (PO) - + - + - + - +
YS1646::rbdB (PO) - + - - - - + +
B
rrbdB Specific IgG (ng/ml)
40000
30000
20000
10000
0
****
YS1646 (PO) - - + - + - - -
rrbdA (IM) - - + - + + - +
rrbdB (IM) - - + - - - + +
YS1646::rbdA (PO) - + - + - + - +
YS1646::rbdB (PO) - + - - - - + +
C
Toxin A Specific IgG Avidity Index (%)
100
80
60
40
20
0
****
**
**
YS1646 (PO) + + - -
rrbdA (IM) + + + +
rrbdB (IM) + - - +
YS1646::rbdA (PO) - - + +
YS1646::rbdB (PO) - - - +
D
rrdbB Specific IgG Avidity Index (%)
100
80
60
40
20
0
***
****
YS1646 (PO) + - -
rrbdA (IM) + - +
rrbdB (IM) + + +
YS1646::rbdA (PO) - - +
YS1646::rbdB (PO) - + +
E
Toxin A Specific IgG1/IgG2c
1000
100
10
1
0.1
0.01
0.001
***
***
**
9/38 34/34 4/6 18/18 42/42 24/24 36/36
YS1646 (PO) - + - + - - -
rrbdA (IM) - + - + + - +
rrbdB (IM) - + - - - + +
YS1646::rbdA (PO) + - + - + - +
YS1646::rbdB (PO) + - - - - + +
F
rrbdB Specific IgG1/IgG2c
1000
100
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0.1
0.01
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**
7/38 34/34 6/12 28/36 24/24 37/37
YS1646 (PO) - + + - - -
rrbdA (IM) - + + + - +
rrbdB (IM) - + - - + +
YS1646::rbdA (PO) + - - + - +
YS1646::rbdB (PO) + - - - + +

Figure 4.2 Multimodal vaccination with YS1646 derivatives expressing rbdA or rbdB generates systemic IgG antibodies. Mice were vaccinated with 3 μg of recombinant antigen (rrbdA/rrbdB) intramuscularly on day 0, with 3 doses of $1x10^8$ cfu of YS1646 delivered orally on days 0, 2 and 4. 4 weeks after vaccination serum was collected and IgG titers were determined by ELISA. **A** Toxin A-specific IgG titers are shown as the mean and standard deviation (SD), a multiple comparison test was used to compare all groups to the PBS group. (n= 25-53, 2-6 repeats) **B** rrbdB-specific IgG titers are shown the same as (**A**). (n=25-47, 2-4 repeats) **C** Toxin A specific IgG avidity index was determined by (antigen-specific IgG concentration remaining after incubation in 6M urea)/(total IgG concentration) x 100%. Only groups that consistently had high IgG titers against Toxin A were tested for avidity. Data is shown as the mean and SD, with a multiple comparison test comparing all groups to the rrbdA/B + YS1646 group. (n=18-42, 3-5 repeats) **D** rrbdB-specific IgG avidity index was determined the same as in (**C**). (n=24-37, 2-3 repeats) **E** Toxin A-specific IgG1/IgG2c ratio was determined by (antigen specific IgG1 titers)/(antigen specific IgG2c titers). A titer below detection was set to 48.75 ng/ml, half of the level of detection. Only mice with detectable titers of at least one of IgG1 or IgG2c were included, the number of mice included per group are indicated above the x axis. The median is the line, and the whiskers show the min and max values. (n=6-42, 2-5 repeats) **F** rrbdB specific IgG1/IgG2c ratio was determined the same as in (**E**). (n=12-38, 2-3 repeats) All panels were analysed using the Kruskal-Wallis test and Dunn's multiple comparison test. *P* values without a bracket are in comparison to the PBS control group. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

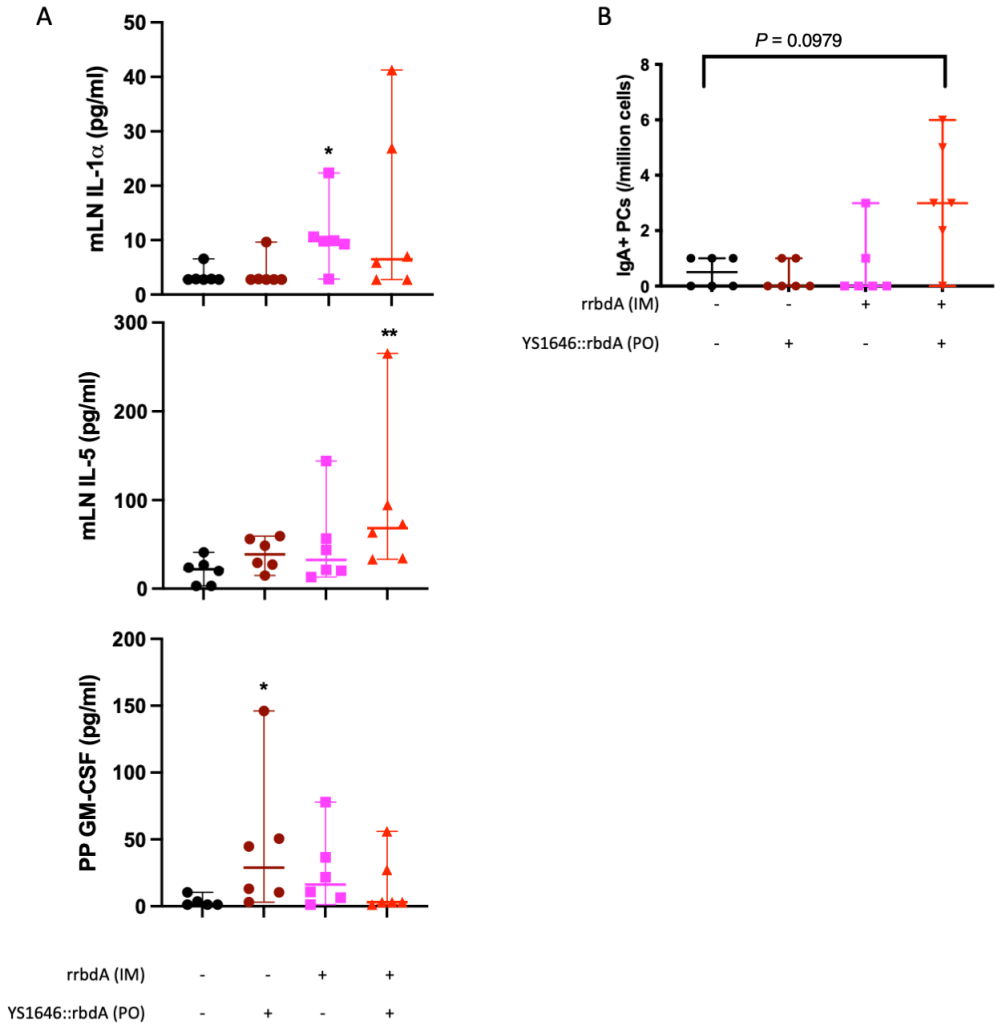


Figure 4.3 Oral delivery of rbdA by YS1646 elicits a mucosal immune response. Mice were vaccinated with 3 µg of recombinant antigen (rrbdA) intramuscularly on day 0, with 3 doses of $1x10^8$ cfu of YS1646 given orally on days 0, 2 and 4. 32 days after vaccination, the mesenteric lymph nodes (mLN) and Peyer's patches (PP) were collected, and cells were isolated. **A** Cells were stimulated for 72h with rrbdA and the supernatant was collected and examined by ELISA to evaluated cytokine and chemokine secretion. (n=5-6, 2 repeats) **B** IgA^+ plasma cells were detected by ELISpot. (n=6, 2 repeats) All data is shown as the median and 95% confidence interval. A Kruskal-Wallis test and Dunn's multiple comparison test to compare all groups to the PBS control was performed. *P* values without a bracket are in comparison to the PBS control group. *, $P < 0.05$; **, $P < 0.01$.

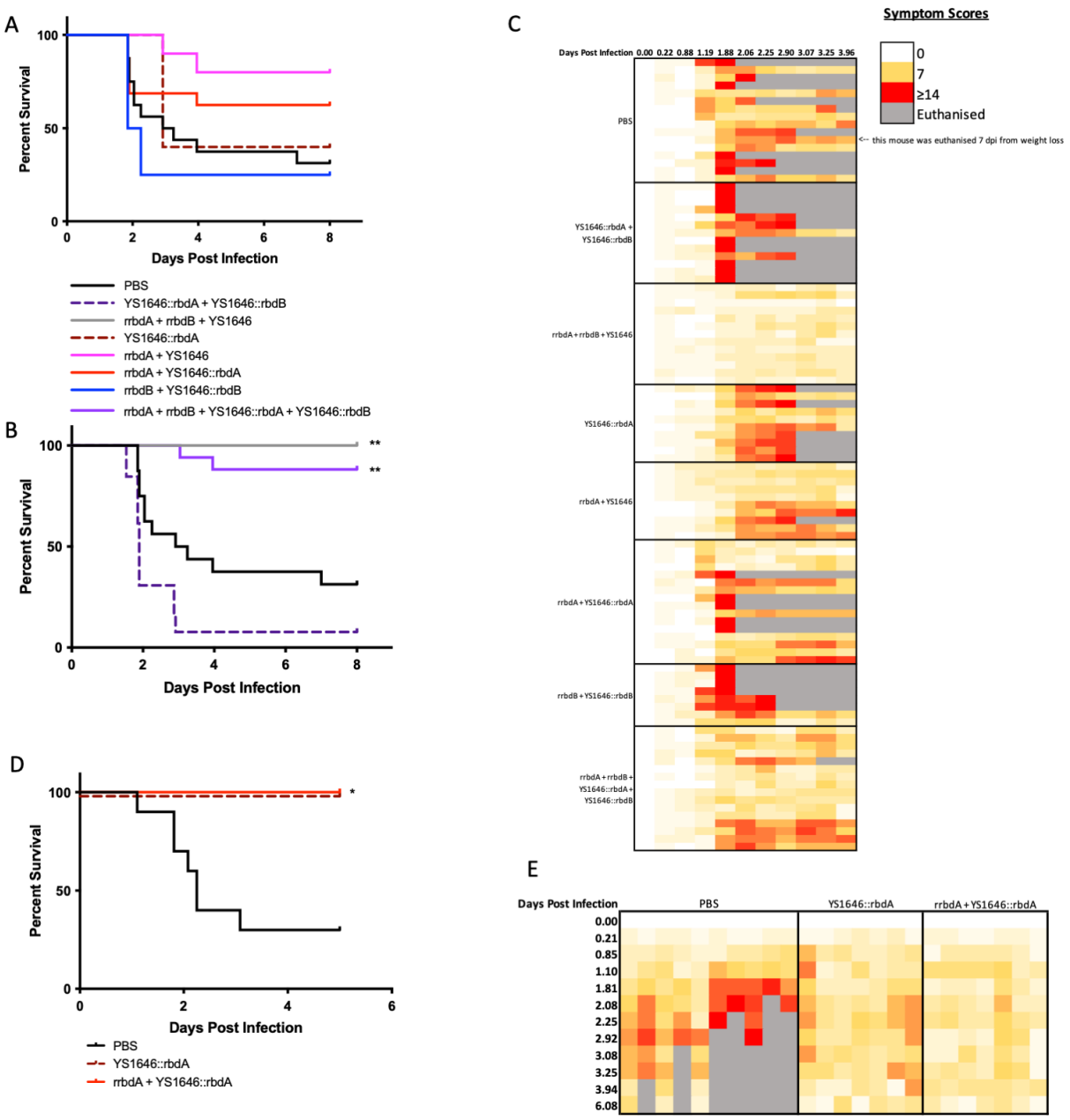


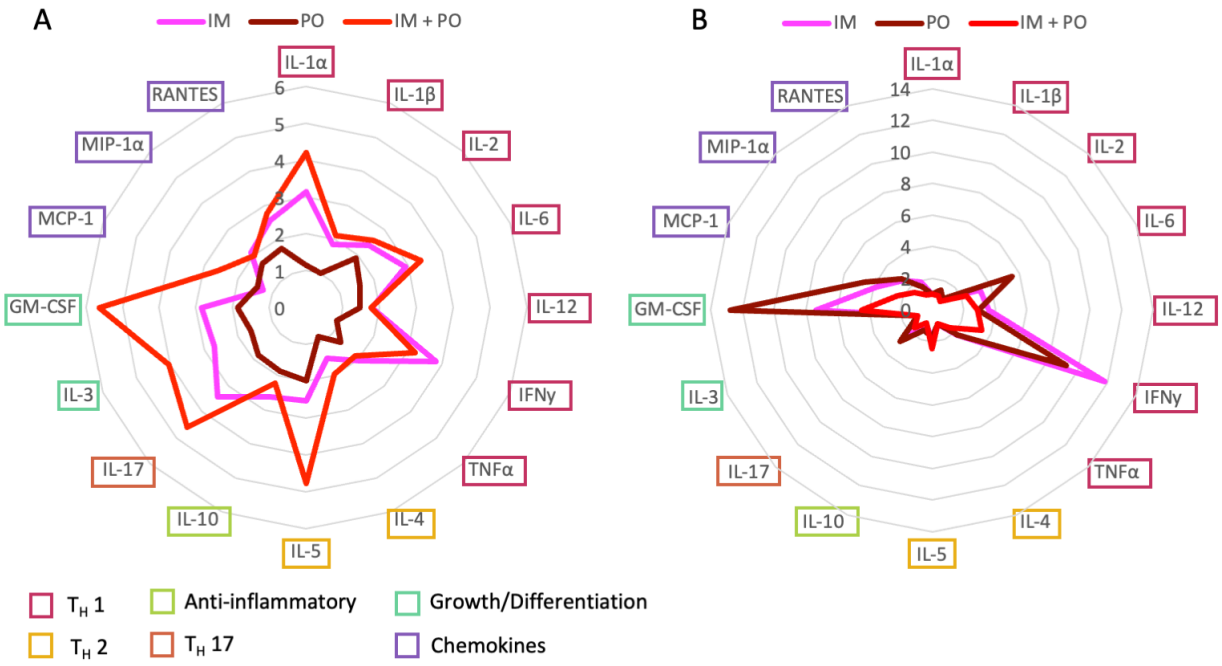
Figure 4.4 High dose vaccination against TcdA provides significant protection against *C. difficile* challenge. **A** Mice from Jackson Laboratories were vaccinated with 3 µg of recombinant antigen (rrbdA/rrbdB) intramuscularly on day 0, with 3 doses of $1x10^8$ cfu of YS1646 delivered orally (PO) on days 0, 2 and 4. At 5 weeks after vaccination, mice were orally challenged with 1370-2500 cfu of freshly cultured *C. difficile*. Mouse symptoms were scored 1-3 times daily by an observer blind to the treatment received. Mice that received a score of 14/20 or above or had over 20% weight loss from their starting weight were at the humane endpoint and were

euthanized. Survival of the groups vaccinated against TcdA or TcdB alone are shown. (n=8-16, 1-2 repeats) **B** Survival of the groups vaccinated against both TcdA and TcdB are shown. (n=13-16, 2 repeats) **C** Symptom scores for all groups are shown. (n=8-16, 1-2 repeats) **D** Mice from Charles River Laboratories were vaccinated with a high dose of vaccine, 10 μg of recombinant antigen (rrbdA) intramuscularly on day 0, with 3 doses of $1x10^9$ cfu of frr_SspH1_rbdA delivered PO on days 0, 2 and 4. At 5 weeks after vaccination mice were challenge with $1.18x10^7$ CFU of freshly cultured *C. difficile*. Survival is shown. (n=7-10, 1 repeat). **E** Symptom scores of mice that received a high dose of vaccine are shown. (n=7-10, 1 repeat). For all survival curves, the log-rank (Mantel-Cox) test was used to compare all groups to the PBS control group. Correction of the *P* value for multiple comparisons was done using the Bonferroni method. *, $P < 0.025$; **, $P < 0.00125$.

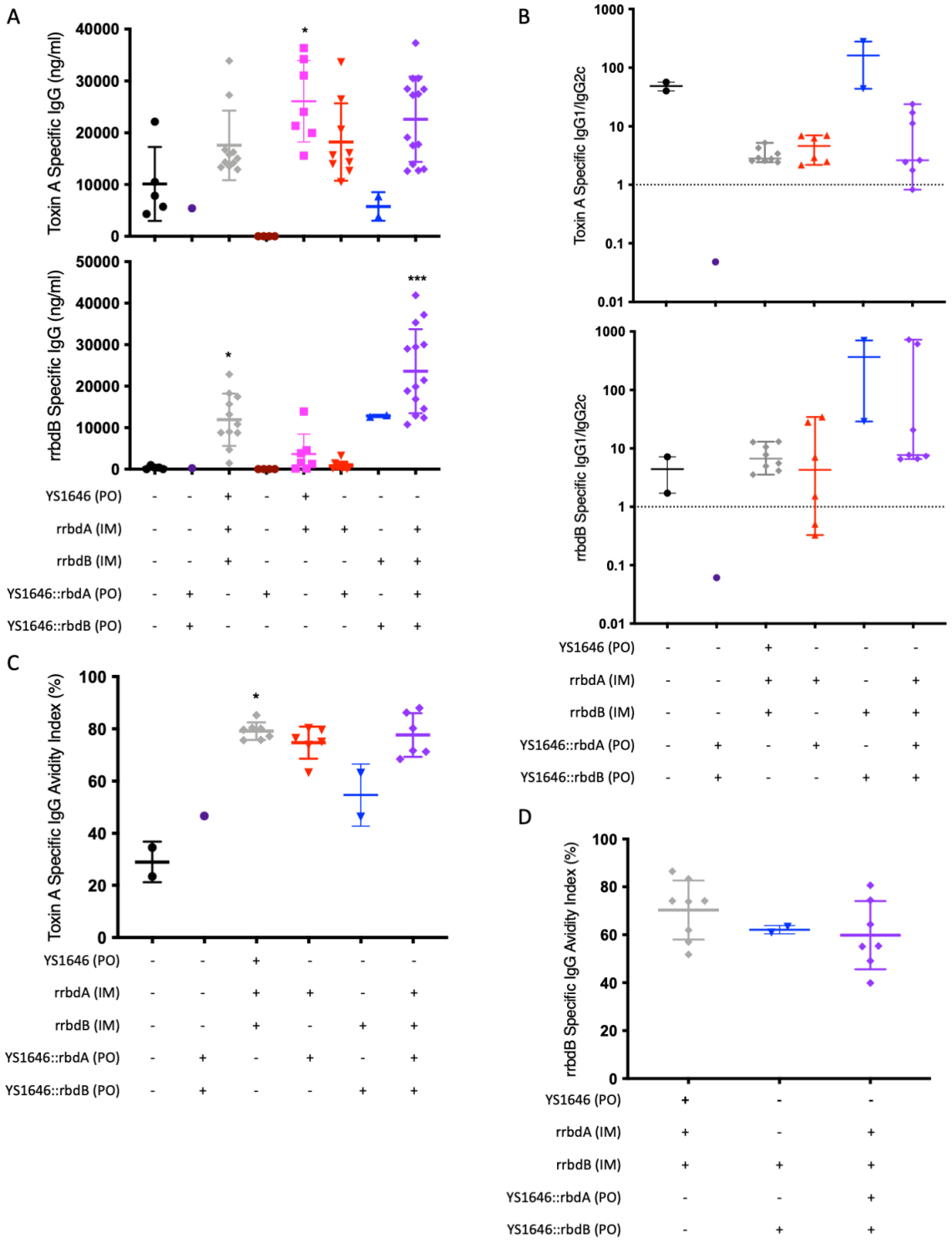
4.13 SUPPLEMENTAL FIGURES AND LEGENDS

Supplemental Table 4.1. Primers used in this study for Tn7 plasmid construction

	Forward Primer (5' --> 3')	Reverse Primer (5' --> 3')	Source
pagC_SspH1_rbdA	CCCGGGCTGCAGGAATTCACGAGGCCCTTTCGTCTTCA	AGGCCTTCGCGAGGTACCGCTTGGCTGCAGATCTTTAACCG	(26)
SspH2_SspH2_rbdA	CCCGGGCTGCAGGAATTCACGAGGCCCTTTCGTCTTCA	AGGCCTTCGCGAGGTACCGCTTGGCTGCAGATCTTTAACCG	(26)
frr	CCCGGGCTGCAGGAATTCCTGCTGCGTAATAACCGTGT	GGATATTAAACATGTTACGAATCCTTGAAAACT	YS1646 genome
SspH1_rbdA	AGGATTCGTAACATGTTTAATATCCGCAATACACAACCTTCT	AGGCCTTCGCGAGGTACCGCTTGGCTGCAGATCTTTAACCG	(26)
pagC_SspH1_rbdB	CCCGGGCTGCAGGAATTCACGAGGCCCTTTCGTCTTCA	AGGCCTTCGCGAGGTACCGCTTGGCTGCAGATCTTTAACCG	(26)
SspH2_SspH2_rbdB	CCCGGGCTGCAGGAATTCACGAGGCCCTTTCGTCTTCA	AGGCCTTCGCGAGGTACCGCTTGGCTGCAGATCTTTAACCG	(26)
frr	CCCGGGCTGCAGGAATTCCTGCTGCGTAATAACCGTGT	AGACAGCATCATGTTACGAATCCTTGAAAACT	YS1646 genome
SspH2_rbdB	AGGATTCGTAACATGATGCTGTCTGGTCAGCG	AGGCCTTCGCGAGGTACCGCTTGGCTGCAGATCTTTAACCG	(26)

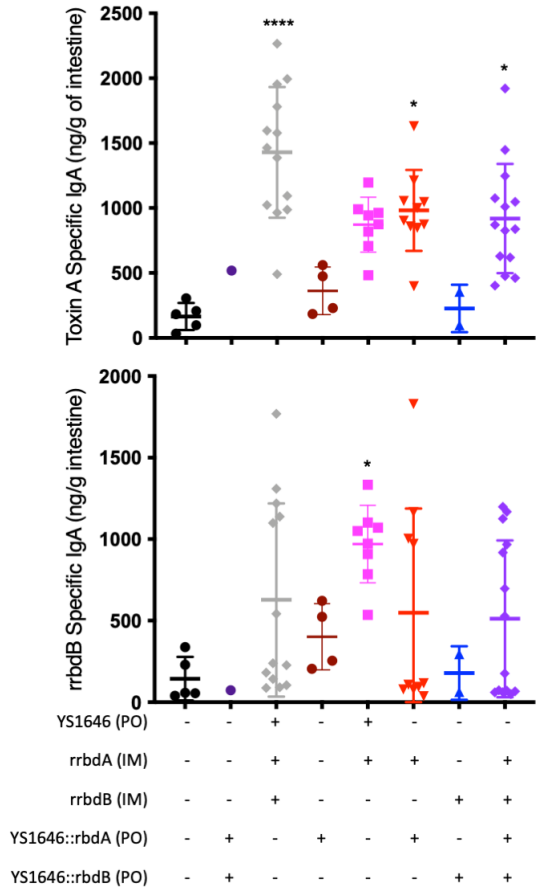


Supplemental Figure 4.1 Vaccination elicits increased cytokine expression in the mesenteric lymph nodes and Peyer's patches. Mice were vaccinated with 3 μg of recombinant antigen (rrbdA) intramuscularly on day 0, with 3 doses of $1x10^8$ cfu of YS1646 given orally on days 0, 2 and 4. 32 days after vaccination, the mesenteric lymph nodes (mLN) and Peyer's patches (PP) were collected, and cells were isolated. Cells were stimulated for 72h with rrbdA and the supernatant was collected and examined by ELISA to evaluated cytokine and chemokine secretion. (n=6, 2 repeats) **A** Cytokine and chemokine secretion from mLN cells is shown. **B** Cytokine and chemokine secretion from cells in the PPs is shown. All data is shown as the fold change of the mean of secreted cytokines and chemokines from the PBS control.



Supplemental Figure 4.2 Vaccination elicits higher systemic IgG titers after challenge, compared to unvaccinated survivors. Mice were vaccinated with 3 μg of recombinant antigen (rrbdA/rrbdB) intramuscularly on day 0, with 3 doses of $1x10^8$ cfu of YS1646 delivered orally

(PO) on days 0, 2 and 4. At 5 weeks after vaccination, mice were challenged with po delivered 1370-2500 cfu of freshly cultured *C. difficile*. Mouse symptoms were scored 1-3 times daily by an observer blind to the treatment received. Mice that received a score of 14/20 or above or had over 20% weight loss from their starting weight were at the humane endpoint and were euthanised. Serum of surviving mice was collected 3 weeks after challenge and IgG titers were determined by ELISA. **A** Toxin A-specific and rrbdB-specific IgG titers are shown as mean with standard deviation (SD) and a multiple comparison test to compare all groups to the PBS control group (n=1-14, 2 repeats). **B** Toxin A- and rrbdB-specific IgG1/IgG2c ratio was determined by (antigen specific IgG1 titers)/(antigen specific IgG2c titers). A titer below detection was set to 48.75 ng/ml, half of the level of detection. Data is shown as the median and 95% confidence intervals, with a multiple comparison test comparing all groups to the PBS control group (n=1-8, 1 repeat). **C** Toxin A-specific IgG avidity index was determined by (antigen-specific IgG concentration remaining after 6M urea incubation)/(total IgG concentration) x 100% . Data is shown as the mean and SD, with a multiple comparison test comparing all groups to the PBS control group (n=1-8, 1 repeat). **D** rrbdB-specific IgG avidity index was determined the same as in (**C**). Only groups with consistently high rrbdB-specific IgG titers were tested for avidity. Data is shown as the mean and SD, with a multiple comparison test comparing all groups to the rrbdA/B + YS1646 group (n=2-8, 1 repeat). All panels were analysed using the Kruskal-Wallis test and Dunn's multiple comparison test. *P* values without a bracket are in comparison to the PBS control group. *, $P < 0.05$; ***, $P < 0.001$.



Supplemental Figure 4.3 Vaccination increases IgA titers in the intestine after challenge, compared to unvaccinated survivors. **A** Mice were vaccinated with 3 µg of recombinant antigen (rrbdA/rrbdB) intramuscularly on day 0, with 3 doses of $1x10^8$ cfu of YS1646 delivered orally (po) on days 0, 2 and 4. At 5 weeks after vaccination, mice were challenged with po delivered 1370-2500 cfu of freshly cultured *C. difficile*. Mouse symptoms were scored 1-3 times daily by an observer blind to the treatment received. Mice that received a score of 14/20 or above or had over 20% weight loss from their starting weight were at the humane endpoint and were euthanised. The small intestine of surviving mice was collected 3 weeks after challenge and IgA titers were determined by ELISA. Toxin A-specific and rrbdB-specific IgA titers are shown as mean with standard deviation (SD) and a multiple comparison test to compare all groups to the PBS control group (n=1-13, 2 repeats). All *P* values are in comparison to the PBS control group. *, $P < 0.05$; ****, $P < 0.0001$.

Chapter 5: General Discussion

Clostridioides difficile infection (CDI) primarily affects the elderly (1). While there has been a decrease in cases of health care acquired CDI (HA-CDI) since 2009, the Center for Disease Control and Prevention (CDC) estimates that the number of elderly living in the United States will double by 2050 (2-5). Community acquired CDI is also on the rise (3, 6, 7). The lack of adequate treatment options for CDI in combination with these other factors highlights the continued need for a vaccine. As discussed in detail in **Section 1.8.1**, 2 vaccines have entered into phase III clinical trials (8, 9). Unfortunately, both failed to meet their primary endpoints. Both of these vaccines had very similar strategies; they used full-length inactivated toxin delivered intramuscularly (IM) to induce a systemic immune response. Since *C. difficile* is an extracellular and non-invasive pathogen that lives in the intestine, we hypothesized that eliciting a mucosal response in the intestine would provide more effective protection in humans.

5.1 MAIN FINDINGS

To address the need for novel vaccination strategies that elicit a mucosal response against *C. difficile,* we repurposed an attenuated *Salmonella enterica* serovar Typhimurium strain, YS1646 to express heterologous antigens. Many successful vaccines on the market target the toxins of toxin-mediated diseases, such as tetanus, diphtheria and whooping cough (10, 11). Several groups have shown that the receptor binding domains (rbd) of Toxins A and B (TcdA and TcdB) of *C. difficile* are immunogenic and that antibodies that bind these regions are toxin neutralizing (12-15). For this reason, we targeted the generation of vaccine candidates that express a portion of the rbd of TcdA and TcdB. In **Chapter 2** we developed 13 plasmid-based, antigen-expressing strains of YS1646. We implemented a multimodal vaccination strategy, in which mice were vaccinated orally (PO) with antigen expressing YS1646 on days 0, 2 and 4 and given an intramuscular (IM) dose of recombinant protein on day 0. Groups vaccinated either IM or PO alone served as controls. IM vaccination was required to generate detectable antigen specific IgG titers in the serum 4 weeks after vaccination (16). IM + PO vaccination against each toxin individually elicited a slight increase in toxin-specific IgA titers in the intestine 5 weeks after vaccination. When we challenged mice 5 weeks after vaccination, all the mice that received antigen IM had 100% survival. The PO only vaccination against both toxins elicited substantial

protection with 82% survival. Mice vaccinated against TcdB IM + PO experienced almost no severe clinical symptoms.

We were interested in the longevity of the responses elicited by the new vaccination described in **Chapter 2**. In the context of Pfizer's recently released phase III clinical trial results, in which patients experienced decreased protective efficacy over the period of 3.5 years, this question takes on added importance (9). In **Chapter 3** we observed that IgG titers were maintained in mice for 6 months, and vaccination either IM + PO or PO only elicited antigen-specific IgA titers that persisted for at least 6 months after vaccination. Upon challenge 6 months after vaccination, all the vaccination strategies provided significant protection. This allowed us to hypothesize that either the multimodal or oral alone vaccination strategies might also generate long lasting responses in humans.

After demonstrating proof-of-concept and the capability of our vaccine candidates to generate long-lasting immune responses using plasmid-bearing YS1646 strains, we turned our attention to developing a vaccine candidate that would be suitable for use in humans. We wanted to develop a vaccine candidate that had stable antigen expression, no mobile genetic elements, and no antibiotic resistance. To do this, in **Chapter 4** we integrated several promoter, secretory signal and antigen sequences into the YS1646 genome (17). Using these candidates, we further investigated the immune responses elicited by vaccination with attenuated *S.* Typhimurium. We observed an increase in antigen specific IL-5 expression in mesenteric lymph nodes (mLN), which was paired with a slight increase in IgA^+ plasma cells in the mLN. Upon challenge with low doses of the chromosmally-integrated (CI) YS1646 strains, IM vaccination against both toxins was required for significant protection. However, using a higher dose of the CI YS1646 strains, PO + IM and PO only vaccination against TcdA provided 100% protection.

5.2 LIMITATIONS OF THE WORK

Although the work done in this thesis generated a novel vaccine candidate against *C. difficile* that is suitable for human use, there are several limitations and important considerations regarding both the work performed and the concept of vaccinating those most susceptible to *C. difficile* infection (CDI) with a live attenuated vaccine.

5.2.1 Relevance of the Mouse Model

Mouse models are used as a fundamental tool in immunology, as they provide a more complicated and reactive system than what can be developed *in vitro*. They are less expensive and easier to house and handle than other rodents, such as rats, hamsters and rabbits (18). There are also many reagents available to study mouse immune responses in depth. However, mice do not always accurately or fully replicate responses seen in humans. A relevant example in the context of this work, is that toxoids administered IM can provide a high level of protection from *C. difficile* challenge in mice (**Chapters 2-4**) (19, 20). As noted above however, this vaccination strategy did not protect humans in phase III clinical trials (8, 9).

5.2.1.1 Use of Salmonella enterica serovar Typhimurium as a vector in mice

S. Typhimurium is so named because it is a mouse pathogen. In mice, wild type *S.* Typhimurium causes a disease that closely resembles *S.* Typhi infection in humans (ie: typhoid fever) (21). It is invasive and crosses the intestinal mucosa without triggering a strong mucosal response followed by dissemination systemically (22). WT *S.* Typhimurium can cause active infections in the spleen, liver, and gall bladder (23, 24). We have observed that YS1646 disseminates to the spleen and liver of mice (unpublished data). We were able to isolate plasmid-bearing YS1646 from the spleens of mice for up to 3 weeks after vaccination. Therefore, in **Chapter 3** we examined the cell mediated memory response in splenocytes.

In **Chapter 4**, we examined the mucosal responses to vaccination in mice. We saw some significant changes in cytokine expression in the mLN and Peyer's patches (PP), namely in IL-5 and GM-CSF. The subtly of the differences we observed, may be due to the nature of how YS1646 interacts with the mouse intestine, as an invasive but attenuated *S.* Typhimurium. In humans, where it may be restricted to the intestinal lumen, intestinal epithelial cells, M cells and mucosal macrophages, we expect to see a more pronounced mucosal response to vaccination (25, 26). There are also several differences between the mouse and human mucosal immune responses that may play a role. As discussed in **Section 1.4.1**, neutrophils play a vital role in the initial response to a gastrointestinal tract infection. However, in the mouse neutrophils account for a much smaller proportion of the white blood cells (wbc). Only 10-30% of wbcs in the blood

of mice are neutrophils, while 50-70% of human wbcs are neutrophils (27, 28). Systemically, mouse and human B cells have many differences in functional capabilities. For example, mouse B cells express TLR4, unlike their human counterparts which allows them to respond to lipopolysaccharide (LPS) in a T cell independent manner (29). Mice also have less circulating IgA in the serum, as most of the IgA produced in mice is specifically targeted to protecting mucosal surfaces (30). The IgA produced by mice is predominantly monomeric, while humans have a mix of monomeric and dimeric IgA. These differences could significantly influence the humoral responses elicited by vaccination in mice and humans. In a review of the differences between intestinal immunity in mice and humans Gibbons and Spencer wrote "the functional outcome may seem the same but the mechanism might be very different" (30). This could be particularly true when discussing the induction of T_H17 responses by either gut-targeting infections or vaccines. T_H17 differentiation in mice can be induced by TGF-β and IL-6, with IL-6 activating STAT3 and RORγT, while TGF-β inhibits the transcription factors for T_H1 and T_H2 responses (31-35). In contrast, TGF-β and IL-6 are insufficient to induce T_H17 cell differentiation in humans in whom IL-1β with IL-6 or IL-23 are necessary (36, 37). These combinations of cytokines also induce T_H17 differentiation in mice, however the cells are often $ROR\gamma t^+$ and $Tbet^+$ leading to IL-17 and IFNγ production (38). Throughout this thesis we have discussed the correlation between IL-17 and IFNγ production in better CDI outcomes in humans (39, 40). While the difference in mechanism exists, if the outcome of IL-17 production in response to *C. difficile* antigens is consistent across species, protection in our mouse model may resemble protection elicited in humans.

5.2.1.2 Clostridioides difficile challenge in mice

Mice are not naturally susceptible to CDI. However, it was determined in the 1980s, that gnotobiotic mice can be easily infected with *C. difficile* (41, 42). Since then, a model using antibiotics to alter the murine gut microbiome prior to infection has been developed (43, 44). The delivery of 6 antibiotics over a 5-day period prior to infection, allows an environmental niche for *C. difficile* colonization to become available. While the number and concentrations of antibiotics required are greater than what is necessary to facilitate infection in humans, this antibiotic-induced susceptibility is a striking similarity between murine and human disease (45). During this work, our lab had significant challenges in infecting mice from one supplier (ie: Charles

River) with a sufficient dose of *C. difficile* to achieve clinical illness and death. Although not proven, we hypothesize that this is due to the colonization of C57BL/6 mice from Charles River Laboratories with the LEM1 strain of *C. difficile* (46). This strain was originally described in 2018, as being endogenous in mice from some animal facilities. While it produces low levels of TcdA, it lacks high virulence in mice and can persist for 4 weeks after inoculation in gnotobiotic and antibiotic-treated mice. Unpublished data from our lab demonstrates that VPI10463 is cleared from the intestines of mice 3 weeks after infection. Mice that have LEM1 in their gut microbiome prior to infection, are protected from CDI with a more lethal strain, as LEM1 is not hindered by the antibiotic cocktail and is able to outcompete other *C. difficile* strains in the mouse intestine (46). When mice from Jackson Laboratories were used, they were much more susceptible to CDI, and required a dose that was 10^4-fold less, to achieve similar level of symptoms and lethality.

One of the major limitations of the mouse CDI model is that TcdA is the more potent toxin in mice than TcdB (47). Several studies have shown that the opposite is likely true in humans. TcdB is 10^2-10^3-fold more toxic to human colonic epithelial cells and $TcdA^-TcdB^+$ pathogenic strains are more commonly identified than $TcdA^+TcdB^-$ strains (48-51). In a clinical trial using a monoclonal antibody targeting TcdA or one targeting TcdB, it was determined that the monoclonal antibody targeting TcdA had no added benefit when delivered in combination with the antibody targeting TcdB (52). This has particular relevance when we compare the protective efficacy of our TcdA and TcdB targeting vaccines in **Chapter 4**. We observed that our vaccine candidates targeting TcdA had higher protective efficacy than our candidates targeting TcdB. Although this could be due to the differences in the promoter and secretory signal sequences or the nature of the antigens, this observation was likely influenced by the different levels of toxicity of TcdA and TcdB in mice.

5.2.2 Alternative Animal Models

There are other animal models that could be used to investigate the immune responses generated by an attenuated *S.* Typhimurium vaccine vector and/or its' protective efficacy against *C. difficile*. A major caveat for all these models is the increased cost for the animals themselves as well as their housing and care. Furthermore, the lack of some species-specific reagents limits

the depth of experiments that can be performed in some of these models. Other limitations as well as the benefits for each model will be discussed below.

5.2.2.1 Rabbits

Historically, rabbits were the first animal model used in immunology studies (53). Due to their intermediate size, they are relatively easy to house, but large enough to allow easy blood sampling and several assays can be performed on the same tissue. One of the interesting advantages of using a rabbit model for vaccination studies with attenuated *S.* Typhimurium, is there is an established gastroenteritis model with wild-type *S.* Typhimurium (54). Oral delivery of *S.* Typhimurium in New Zealand white rabbits causes diarrheal disease in a dose dependent manner. However, at higher doses, the *S.* Typhimurium disseminates and colonizes the liver and spleens of infected rabbits. While the rabbit immune system more closely resembles humans than that of mice, there are some significant differences in the mucosal responses. Most importantly, rabbits have 11 different IgA subclasses that are expressed at mucosal surfaces (55). While the mucosal response to vaccination in rabbits may not perfectly mimic the response in humans, it would be interesting to examine the responses in an intestinally restricted model. Although rabbits are not commonly used for research in responses to *C. difficile*, they are susceptible to CDI (56-58).

5.2.2.2 Syrian Hamsters

Syrian hamsters are the most commonly used alternative model for CDI (56) because of their extreme susceptibility to CDI. After antibiotic treatment, exposure to 1 cfu of *C. difficile* can lead to fatal disease (59). Hamsters can also be infected without antibiotic treatment (60). However, contamination control is vital when performing CDI experiments with hamsters. While the hamsters' absolute susceptibility does allow for easy establishment of a fatal challenge model, humans are not as susceptible to CDI. The hamster model provides a possibly insurmountable challenge, giving false negative results. Hamsters have not been used in either infection or vaccination studies involving *S.* Typhimurium and are rarely infected by wild type *S.* Typhimurium outside of the lab setting (61).

5.2.2.3 Piglets

Pigs are susceptible to *S.* Typhimurium colonization and can experience symptoms from a wild-type infection or remain asymptomatic carriers (62). *S.* Typhimurium burden is highest in pigs in their tonsils, intestines, and gut-associated lymphoid tissues (GALT) (62, 63). Unlike humans, the upper gastrointestinal tract and mucosal surfaces may play an important role in transmission and infection (62). At high doses, *S.* Typhimurium can spread systemically in pigs (64). There are several other differences between the mucosal immune response in pigs and humans that could affect the responses elicited by our vaccine. Porcine intestinal epithelial cells (IECs) do not express MHC Class II and are therefore unable to present antigen as non-professional antigen presenting cells (APCs) (65). Pigs also have one extremely long, continuous ileal PP, rather than several distinct PPs (66).

Piglets are susceptible to CDI, leading to the development of two different neonatal piglet models of CDI (67-71). The less expensive option involves treating piglets with vancomycin 2 days after birth to ablate any previous *C. difficile* colonization and then infecting them around day 7 after birth (67). Neonatal piglets are only susceptible to *C. difficile* colonization/infection for the first week or two after birth (70). This poses a serious limitation for the ability to vaccinate and then challenge in this model. In addition, maternal antibodies do not cross the porcine placenta, so vertical transmission of immunity would require the piglets to suckle after birth and potentially be exposed to *C. difficile* and other pathogens by their mothers (65). The more expensive model uses gnotobiotic piglets (71). In this model, piglets are delivered by caesarean section and maintained in sterile incubators where they are infected. This model has clinical manifestations that are quite similar to CDI in humans, including gastrointestinal and systemic symptoms, mucosal legions, pseudomembrane colitis, increased IL-8 in the gut and protection by monoclonal antibodies targeting TcdB but not TcdA (52, 71, 72). It is hypothesized that he gnotobiotic state of the piglets extends the window of susceptibility to CDI, perhaps allowing for vaccination prior to challenge. However, most groups using this model infect piglets around day 5 after birth.

5.2.3 Safety concerns in live-attenuated vaccination of the elderly

A strong priority when developing a preventative intervention such as a vaccine, is the safety and tolerability of the product. For the same reasons the elderly are most at risk for CDI, they also have a higher risk of not responding to or having adverse events after vaccination with a live-attenuated *S.* Typhimurium. Immunosenescence in the context of CDI was discussed in detail in **Section 1.5.1**. The decreased function of neutrophils and dysregulated cytokine and chemokine production experienced during immunosenescence could result in some older individuals being unable to control a YS1646 vaccination, leading to gastroenteritis. The demonstrated susceptibility of YS1646 to CO_2 concentrations in human blood allows us to hypothesize that YS1646 would have extreme difficulty in causing sepsis (73). A recent study observed decreased mucosal responses in the elderly after vaccination with Ty21a (74). They identified a decreased frequency of *S.* Typhi-specific $CD4^+$ T effector memory cells (T_{EM}) and decreased IL-17A and IL-2 production by $CD4^+$ T resident memory cells (T_{RM}) and $CD8^+$ T cells in the terminal ileum lamina propria. While these caveats highlight the need to proceed cautiously as we eventually move towards vaccinating the elderly, we feel that the urgent need for a novel vaccination strategy is sufficient to drive forward with this candidate live-attenuated vaccine.

5.3 FUTURE PERSPECTIVES

5.3.1 Targeting the hypervirulent NAP1

The hypervirulent strain of *C. difficile* that causes ~30% of cases bears several names including NAP1 (75). Several differences in the regulation and the sequence of TcdB contribute to the increased virulence of NAP1 strains (76, 77). The rbd of $TcdB_{NAP1}$ is antigenically distinct from most historical strains (78, 79). Therefore, it is possible that our vaccine candidates would not protect against CDI caused by NAP1 strains. While NAP1 strains do not represent the majority of CDI cases, it has been shown that rates of HA-CDI are positively correlated with the proportion of cases that are $NAP1^+$ (80). To address this concern, two students in our lab have collaborated to develop a YS1646-based vaccine targeting the glucosyltransferase domain (gtd) of $TcdB_{NAP1}$ which has a highly-conserved sequence with historical strains (78). Our lab has generated plasmid-based, gtd expressing strains of YS1646 and developed a NAP1 challenge model in mice, however we are currently working on demonstrating the immunogenicity of the

gtd antigen (unpublished data). Once the immunogenicity of the antigen is demonstrated, as it has been shown by other groups, we will examine the ability of this vaccine to protect against both NAP1 and VPI10463 strains, and its ability to complement the rbdB vaccines we have already developed (81, 82). The limitations of delivering multiple antigens with a live-attenuated vaccine will be discussed in detail in **Section 5.3.2.1.1**. However, if the gtd vaccine candidate is promising, the gene coding for this antigen will be chromosomally integrated into YS1646, so it can be further tested in non-clinical studies and clinical trials.

5.3.2 Moving towards human use

Taking this project forward means moving towards licensure and human use. Below, both considerations for use in humans and a possible pathway to licensure will be discussed.

5.3.2.1 Considerations

5.3.2.1.1 Multiple Antigens

This project developed vaccine candidates that target two related but distinct antigens. Moving into clinical trials, it is unclear if targeting TcdB alone would be sufficient to provide protection against CDI. To date, all Phase III clinical trials for *C. difficile* vaccines have targeted both TcdA and TcdB (8, 9). It was determined that a monoclonal antibody targeting TcdA did not provide additional advantages when combined with a monoclonal antibody targeting TcdB (52). However, the licensed monoclonal antibody targeting TcdB is not sufficient to clear a CDI infection and is used in combination with antibiotic therapy to reduce recurrence (83). Vaccinating against two antigens complicates studies in several ways. Delivering two antigen-expressing YS1646 strains could require doubling the dose to elicit similar immune responses to both antigens, which would likely increase the risk of adverse events. One of the two strains could also outcompete the other in the gut, leading to a stronger response against one antigen. In **Chapter 4**, we observed that a chromosomally integrated YS1646 expressing rbdB outgrew a strain expressing rbdA. We also observed decreased IgG avidity in mice vaccinated against both toxins compared to mice vaccinated against just TcdA. However, the decreased avidity was also present mice vaccinated IM only, suggesting the differences observed are antigenic in nature rather than due to YS1646 competition in the intestine. Another graduate student in our lab, in collaboration with the Dozois lab, has been working on chromosomally integrating repeated

sequences for a single antigen by sequential insertions (ie: multi-copy chromosomal integration). One of the challenges with this method of integration occurs when removing the chloramphenicol cassette after the second insertion. The recombinase flippase (Flp) tends to use the FRT site left in the first insertion, leading to the removal of the first insert with the chloramphenicol resistance cassette. This can be avoided by inserting the double sequence at one time. However, the increased length of the insertion makes this approach more challenging. Once this protocol has been optimized, it should be possible to generate a strain of YS1646 that expresses both rbdA and rbdB (or both the rbdB and the gtd genes). While this would solve some of the potential issues mentioned above, the possibility that one of the antigens would be immunodominant would remain. All these considerations must also be taken into account when we contemplate the addition of the gtd antigen to our vaccine cocktail.

5.3.2.1.2 Storage

A benefit to using a live-attenuated *S.* Typhimurium vaccine vector is the ease with which it can be stored. The Ty21a vaccine is stored lyophilized between 2-8ºC (84). This is considerably easier and less costly to maintain than products that require storage below -20ºC or -70ºC. We have lyophilized our antigen-expressing YS1646 and it is stable at room temperature for two months after lyophilization (unpublished data). Three months after lyophilization, we observed a log decrease in the concentration of the bacteria. This study is ongoing and needs to be repeated at 4ºC, but it suggests that a live-attenuated YS1646 vaccine would not require freezing for proper storage. Recombinant protein vaccines are generally also stable at 2-8ºC (10, 85).

5.3.2.1.3 Multimodal vaccination

We hope that PO vaccination with the antigen expressing YS1646 strains will be sufficient to protect from CDI. However, there is a strong possibility that IM vaccination with a recombinant antigen may also be necessary for either rapid protection and/or effective protection. While the multimodal vaccination strategy poses additional Chemistry-Manufacturing-Control (CMC) and regulatory challenges, if the vaccine proves to be highly effective, its licensure is still possible. Even if multimodal vaccination is necessary, administration of the IM and PO doses would be relatively simple and would only require one

visit with a healthcare worker (HCW). In that visit the HCW would administer the IM dose and supervise administration of the first PO dose. The patient could then take the remaining PO doses at home. This is currently the strategy for the Ty21a vaccine (84).

Multimodal vaccination increases the costs and complicates toxicity testing and manufacturing of the vaccine. Each component would need to be tested for toxicity individually and in combination with the others. With the potential need for 2 antigens, delivered by two methods, the increased cost is significant. While production of recombinant proteins and a live-attenuated vaccine is simple enough, requiring both to be manufactured does require two separate protocols to be developed and optimized.

While there are currently no multimodal vaccines on the market, which may complicate the path to licensure, regulators have accepted increased flexibility for vaccine mixing and matching throughout the COVID-19 pandemic (86, 87). Considering the current lack of effective vaccines against CDI, if multimodal vaccination proves to be the most effective strategy, it should be possible to achieve licensure.

5.3.2.2 Pathway to Licensure

5.3.2.2.1 Good manufacturing practices

Good manufacturing practices (GMP) are required by almost all countries for the production of biological products (88). The World Health Organization (WHO) has been publishing GMP Guidelines since 1992 (89). Many countries including Canada and the USA have their own GMP regulations as well. These regulations are put in place to ensure products have consistent quality and safety. GMP manufacturers are certified in Canada (90). Before testing of our vaccine candidate(s) can advance to human trials, we would need to develop a scaled-up protocol that follows GMP regulations. This could be done in a “GMP-like” facility, a facility that is not certified, but follows GMP regulations. Once this protocol is in place, it can be transferred to a GMP facility and optimized under full GMP.

5.3.2.2.2 Toxicity Testing

Prior to beginning a Phase I clinical trial, our vaccine(s) would need to undergo toxicity testing. YS1646 itself has already undergone such testing as well as a phase I clinical trial in which it was deemed to be safe in humans after intravenous injection of doses up to 10^8 cfu (91). It is possible that we could use the previously established safety record of YS1646, to limit the toxicity tests we would have to perform. However, for our multimodal vaccination strategy, we would need to test the toxicity of our IM delivered recombinant protein alone and in combination with PO delivered YS1646.

Toxicity testing includes examining the toxicity of the reagent (mortality, clinical signs, body weight gain or loss) and local tolerance to administration (erythema and oedema at the injection site) in at least one relevant animal model (92). If more than one dose will be delivered to humans, multiple dose testing is typically required. These studies must be performed under good laboratory practices (GLP).

5.3.2.2.3 Phase I/II clinical trials

A phase I clinical trial is used to determine the safety of a drug/vaccine in humans. Participants would be young, healthy adults. The first participants would be vaccinated with a low dose of the vaccine and monitored for adverse events. Since our PO delivered vaccine is alive, we would also need to monitor bacterial shedding and clearance. Fortunately, we do have a fail-safe, in that our antigen-expressing YS1646 is susceptible to antibiotics. If a participant were to become seriously ill or was unable to clear the bacteria after several days, we would be able to administer ampicillin to kill the YS1646. This would still be considered a major adverse event, since our vaccine would probably not be licensed if it required the use of antibiotics for clearance in any substantial proportion of individuals. After successful demonstration of safety at a low dose in healthy adults, the phase I clinical trial would perform dose-ranging studies and further safety studies in the elderly.

Considering the lack of concrete evidence of correlates of protection for CDI, evaluating the immunogenicity of our vaccine in humans could be challenging. We could examine antigen-specific IgG and IgA in the serum, as well as antigen-specific T cells in the blood. IgA in the

feces could also be analysed. Examining mucosal responses via a colonic biopsy would likely be too invasive for an early-phase clinical trial. If the vaccine is eventually licensed, or in a Phase III clinical trial, it would be very interesting and informative to recruit participants prior to a scheduled screening colonoscopy. A biopsy could be collected and then tissue resident T cells could be analyzed for antigen-specific cytokine secretion. Antigen-specific IgA^+ B cells and IgA in the tissue could also be analysed.

Developing an orally delivered, live-attenuated vaccine requires walking a fine line when it comes to immunogenicity. The vaccine needs to be sufficiently attenuated to not cause serious adverse events. However, it also needs to be sufficiently "pathogenic" to overcome intestinal mucosal tolerance and elicit an effective immune response. If, in a phase I clinical trial, it is deemed that YS1646 is either too pathogenic or not immunogenic enough, we have several options for changing the vaccine vector. If YS1646 is too pathogenic, the mutations used to attenuate Ty21a are now off patent and could be used to further attenuate YS1646. If YS1646 is not immunogenic enough, we have access to the parent strain of YS1646, YS72, and other strains that were further attenuated from YS72 (93). These strains could potentially be more immunogenic in people. However, the redevelopment of our PO vaccine candidate, would mean we would need to redo all the pre-clinical work, including toxicity testing. Without the already established safety record of YS1646, new YS72-based strains (or further attenuated YS1646 strains) would require more extensive toxicity testing than our current vaccine candidates.

5.3.2.2.4 Phase III clinical trial

One of the most expensive steps in the pathway to licensure is the phase III clinical trial. Sanofi Pasteur's *Cdiffense*™ recruited 9,302 patients, while Pfizer's CLOVER recruited 17,571 patients. Due to the rate of CDI, which has been decreasing since these trials started, such large study populations were required to have enough power to assess vaccine efficacy. One of the benefits of our vaccine strategy, may be its ability to elicit a rapid immune response, possibly with one week of vaccination. Exploiting this advantage, we could recruit patients who have recovered from primary CDI into our Phase III clinical trial and monitor recurrences for 12 months after vaccination. This would greatly reduce the number of participants we would need to recruit, as up to 35% of patients who receive standard treatments experience recurrent CDI (94).

5.4 CONCLUDING REMARKS

In comparison with other drug discovery approaches, repurposing failed pharmaceuticals has a good rate of success (95). In this work, we took a failed cancer therapeutic and repurposed it as a vaccine against *C. difficile*. We moved from proof-of-concept with a plasmid-based system, to a stable chromosomally integrated drug candidate. We have demonstrated that a multimodal vaccination strategy elicits both systemic IgG antibodies and mucosal responses against *C. difficile* antigens. This vaccination strategy can provide 100% protection to mice from lethal challenge. At high doses with some YS1646 stains, PO vaccination alone can also entirely protect mice from *C. difficile* challenge. This novel vaccination strategy is able to elicit protective responses in a short period of time, raising the possibility that it could be used after primary CDI to prevent recurring CDI. While considerable work remains to be done to bring a YS1646-based vaccine to licensure, the candidate vaccines described in this work have shown promise and could eventually be vital tools in the prevention of CDI.

5.5 REFERENCES

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