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An Integrated Approach to Oral Vaccination against Enteric Pathogens

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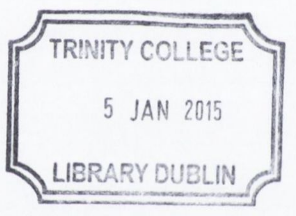
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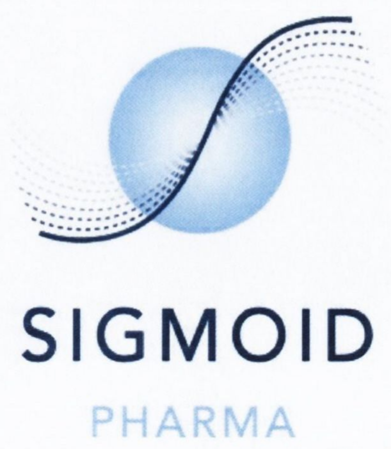

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Christopher Davitt

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Abstract

While the majority of human pathogens infect the body through mucosal sites, most licenced vaccines are injectable. In fact the only mucosal vaccine that is widely used for infant and childhood vaccination programs is the oral polio vaccine (OPV) developed by Albert Sabin in the 1950s. While oral vaccines against Cholera, rotavirus and *Salmonella typhi* have been licenced, the development of vaccines against other enteric pathogens has been slow and challenging. Mucosal vaccines elicit protective immunity at the gut mucosa, in part via antigen-specific secretory immunoglobulin A (SIgA). This explains the low efficacy of injectable cholera vaccines, which have been abandoned in favour of oral cholera vaccines (OCVs). However, oral vaccines face many challenges including passage through the acidic environment of the stomach followed by the proteolytic environment of the duodenum which can lead to the destruction of vaccines. The gut innate immune system is also difficult to activate due to its predisposition towards hyporesponsiveness and tolerance. Lastly, adjuvants such as alum, incorporated in injectable vaccines to enhance their immunogenicity are lacking for the oral route.

Enterotoxogenic *Escherichia coli* (ETEC) are the cause of an acute watery diarrhea and are spread through the consumption of contaminated food and water. The global disease burden of ETEC is difficult to measure, but the bacterium is estimated to cause over 210 million cases and 380,000 deaths annually, mostly in children. ETEC is also a leading cause of Traveller's Diarrhoea in visitors to endemic regions. Recently, oral vaccine efforts against ETEC have focused on the generation of whole cell killed (WCK) bacteria expressing colonisation factor antigens (CFAs), a family of molecules that mediate the attachment of ETEC bacteria to intestinal epithelial cells (IECs) making them an essential step in pathogenesis. CFA/I has been identified as one of the most widely expressed CFA molecules across several regions. A strategy to over-express these on non-toxic *E.coli* yielded a promising WCK antigen. The mucosal immunogenicity of this WCK antigen was further enhanced when co-administered orally with the iNKT activating ligand α-galactosylceramide (α-Galcer). The effectiveness of this candidate oral ETEC vaccine (OEV) was potentiated further when encapsulated in an oral delivery vehicle, the Single-Multiple Pill® (SmPill). By protecting the vaccine from the acidic environment of the stomach, targeting its release in the intestine and potentiating the immunogenicity of the formulation with a suitable adjuvant it was shown that one can greatly enhance the oral efficacy of a candidate OEV in driving intestinal immune responses.

Similarly to ETEC, Cholera is a severe diarrhoeal infection. The diarrhoea caused by cholera however, is much more severe than ETEC. Causing between 3-5 million cases and resulting in over 100,000 deaths annually, cholera is most devastating during an epidemic, such as the ongoing outbreak in Haiti. Although OCVs exists, eliciting SIgA-mediated immunity against bacterial LPS and cholera toxin (CT), the active cause of the diarrhoea, the protective efficacy of these vaccines is often low. Utilising the integrated approach outlined above, α-Galcer in SmPills was shown to enhance anti-CTB and anti-LPS IgA responses compared to the clinically licenced OCV Dukoral®. Furthermore, an interesting contrast was observed between CT and α-Galcer with regard to the differential adjuvanticity these displayed for soluble and particulate antigens.

One of the greatest challenges facing the development of effective oral adjuvants is an incomplete understanding of the mechanisms underlying vaccine-mediated protection in the gut. IL-17 was recently identified as an important cytokine regulating intestinal SIgA transport. IL-17R deficiency resulted in reduced antigen-specific intestinal IgA levels following vaccination. Mice lacking IL-17R also succumbed to oral CT challenge after vaccination, while wild-type mice were protected following vaccination with an efficacious CTB based vaccine.

Overall these findings highlight the need to design integrated approaches that address the challenges of oral vaccination collectively rather than in isolation. Furthermore, improved understanding of the mucosal immune effector mechanisms is essential to facilitate novel approaches to address these challenges and development new and improved oral vaccines.

Abbreviations

ADP	adenosine diphosphate
AHR	aryl hydrocarbon receptor
AID	activation-induced cystidine deaminase
AIEC	adherent invasive *E.coli*
AMP	adenosine monophosphate
APC	antigen-presenting cell
APRIL	a proliferation-inducing ligand
APS	ammonium persulphate
ASC	antibody secreting cell
AUC	area under the curve
BAFF	B cell activating factor of the tumor-necrosis factor family
BALT	bronchus-associated lymphoid tissue
BCR	B cell receptor
BSA	bovine serum albumin
C domain	constant domain
cAMP	cyclic adenosine monophosphate
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	cluster of differentiation
CD40L	CD40 ligand
CDC	centres for disease control
CDR	complementarity-determining region
CE	caecum
CF	colonisation factor
CFA	colonization factor antigen
CFTR	cystic fibrosis transmembrane regulator
cGMP	cyclic GMP
cGMP	cyclic guanine monophosphate
CNS	central nervous system
cRPMI	complete roswell park memorial institute
CSR	class switch recombination
CT	cholera toxin
CTA	cholera toxin A subunit
CTB	cholera toxin B subunit
CTL	cytotoxic T lymphocyte
DAEC	diffusely adherent *E.coli*
DAMP	danger-associated molecular pattern
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
dmLT	double mutant LT
E.coli	*Escherichia coli*
EAEC	enteroaggregative *E.coli*
EDTA	ethylenediaminetetraacetic acid
EHEC	enterohaemorrhagic *E.coli*
EIEC	enteroinvasive *E.coli*
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic *E.coli*
ER	endoplasmic reticulum

ERAD	ER-associated degradation
ETEC	enterotoxigenic *E.coli*
FAE	follicle-associated epithelium
FCS	fetal calf serum
FDA	food and drug administration
FDC	follicular dendritic cell
FK	formalin killed
FK.ETEC	formalin-killed CFA-I overexpressing *E.coli* whole cell bacteria
GALT	gut associated lymphoid tissue
GC	germinal centre
GC-C	guanylate-cyclase-C
G-CSF	granulocyte-stimulating colony factor
GIT	gastrointestinal tract
GM1	monosialotetrahexosylganglioside 1
GMP	good manufacturing practise
$G_{s\alpha}$	guanine nucleotide binding protein α-subunit
GTP	guanine triphosphate
HCl	hydrogen chloride
HepB	hepatitis B
HEV	high endothelial venule
HGT	horizontal gene transfer
HIV	human immunodeficiency virus
HPV	human papillomavirus
HUS	hemolytic uremic syndrome
i.p	intra-peritoneally
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IEL	intra-epithelial lymphocyte
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IL-17R	IL-17 receptor
IL-23R	IL-23 receptor
ILC	LTi-like innate lymphoid cell
ILF	isolated lymphoid follicle
iNKT	invariant natural killer T
iNOS	inducible nitric oxide synthase
IPV	inactivated polio vaccine
IQ	intelligence quotient
IRIV	immunopotentiating reconstituted influenza virosome
ISCOM	immune stimulating complex
JS1569	*Vibrio cholerae* strain expressing Inaba LPS
KO	knockout
LI	large intestine
LP	lamina propria
LPS	lipopolysaccharide
LSI	lower small intestine
LT	heat-labile enterotoxin
LTB	B subunit of heat-labile enterotoxin
LTi	lymphoid tissue inducer

M cells	microfold cell
MADCAM-1	mucosal addressin cell adhesion molecule-1
MAIT	mucosal-associated invariant T
MALT	mucosa-associated lymphoid tissue
mH_2O	millipore water
MHC	major histocompatibility complex
MLN	mesenteric lymph node
mNKT	mucosal natural killer T
MNP	mononuclear phagocyte
mORC-Vax^{TM}	modified ORC-VAX^{TM}
MP	microparticle
MPLA	monophosphoryl lipid A
MS1346	*Vibrio cholerae* strain expressing Inaba and Ogawa LPS
MyD88	myeloid differentiation primary response gene 88
Mφ	macrophage
NALT	nasopharynx-associated lymphoid tissue
NK	natural killer
NK	natural killer
NKT	natural killer T
NLR	nod-like receptor
OCTC	oral cholera toxin challenge
OCV	oral cholera vaccine
OD	optical density
OEV	oral ETEC vaccine
OMV	outer membrane vesicle
OPD	*o*-phenylenediamine
OPV	oral polio vaccine
OVA	ovalbumin
PAI	pathogenicity island
PAMP	pathogen-associate molecule pattern
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with tween 20
pCTB	peptide CTB
PEG	polyethylene glycol
Perfext	perfusion-extraction
pIgR	polymeric Ig receptor
PKA	protein kinase A
PLG	poly(lactide-co-glycolide)
PLGA	poly(lactic-*co*-glycolic acid)
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethanesulfonylfluoride
PP	peyer's patch
PRR	pattern recognition receptor
RA	retinoic acid
RA	retinoic acid
rCTB	recombinant CTB
RLR	rig-like receptor
RNA	ribonucleic acid
ROR	RAR-related orphan receptor
RPMI	roswell park memorial institute

S1P	sphingosine 1-phosphate
SBC	sodium bicarbonate
SC	stromal cell
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SED	subepithelial dome
SFB	segmented filamentous bacteria
SHM	somatic hypermutation
SIgA	secretory immunoglobulin A
SmPill	single-multiple pill®
SPF	specific pathogen free
ST	heat-stable enterotoxin
STAT	signal transducer and activator of transcription
STEAEC	shiga toxin (Stx) producing enteroaggregative *E.coli*
STI	soybean trypsin inhibitor
Stx	shiga toxin
TCR	T cell receptor
TEMED	tetramethylethylenediamine
TFH	follicular helper T cell
TGF-β	transforming growth factor beta
TGF-βR	transforming growth factor beta receptor
TGN	trans golgi network
Th	T helper cell
TLR	toll-like receptors
TNF-α	tumor necrosis factor alpha
T_{reg}	regulatory T cell
tRNA	transfer RNA
TSLP	thymic stromal lymphopietin
UEA-1	*Ulex europaeus* agglutinin I
USI	upper small intestine
V domain	variable domain
V.cholerae	*Vibrio cholerae*
VAPP	vaccine-associated paralytic poliomyelitis
VLP	virus-like particle
WCK	whole cell killed
WHO	world health organisation
α-GalCer	alpha-galactosylceramide

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Chapter One

Introduction

1. Introduction

1.1 – The immune system: a brief introduction

Throughout their lives, vertebrates are constantly challenged by microbial and cellular threats that can lead to disease and death. In order to survive this constant environmental pressure, vertebrates, very early in their evolution developed an immune system. The immune system is a complex and diverse collection of cells and organs distributed throughout the body that eliminate exogenous and endogenous threats from the host.

In order to deal with an ever changing spectrum of potential threats, the immune system evolved into two distinguishable sub-systems, differentiated by their respective levels of detection and effector specificity. The innate immune system contains a limited number of receptors, while the adaptive immune system contains a highly specific, extremely variable repertoire of receptors. Although the receptors of the innate system are fewer and less specific than those of the adaptive system, they are constitutively expressed and can respond rapidly when activated. Effectively the innate immune system acts as a sentinel, that can very readily detect and respond to threats and quickly activate and instruct the adaptive system to mount the most effective response against the particular pathogen, allowing for clearance, healing, and the generation of future protection, or immunity. These two arms of the immune system will be briefly introduced, with their specific relevance to intestinal immune responses being reviewed in greater detail.

The innate immune system has been determined to be the evolutionary product of the immune system of the early multicellular organisms. The innate system together with the anatomical barriers that isolate organs and internal tissue from the environment provide a frontline level of defence.

Pathogens can trigger the initiation of an innate immune response in minutes. The nature of this innate initial effector response depends entirely on the innate immune cells which the pathogen first encounters. Phagocytes such as neutrophils, macrophages and dendritic cells (DCs) often initiate complex innate signalling pathways on encountering a pathogen, which leads to the production of effector molecules including cytokines. These cytokines are often associated with inflammation, and are so deemed pro-inflammatory and include tumor necrosis factor alpha (TNF-α) and interleukin (IL) -1. However, re-exposure to a previously encountered threat will not lead to an enhanced immune response.

The limitation of the innate immune system is that it expresses a defined repertoire of genomically encoded receptors often referred to as pathogen recognition receptors (PRRs) which recognize distinct pathogen associated molecule patterns (PAMPs) on invading organisms or endogenous danger-associated molecular patterns (DAMPs). PRRs are a class of functionally and structurally diverse receptors that mediate the innate and subsequently the adaptive immune response. PRRs act as pathogen sensors that initiate the clearance of the pathogen and the establishment of adaptive immunity. PAMPs constitute a limited number of unique structures, such as bacterial peptidoglycan and viral double-stranded RNA that are evolutionarily conserved microbial and cellular components vital to the survival or pathogenicity of the microorganism. Both PAMPs and DAMPs are signals that indicate that the body is under some form of insult, and the specificity of the cognate receptor will indicate to some extent the nature of the insult, be it viral, bacterial etc. There exists two functionally different classes of PRRs; signalling and non-signalling. Signalling PRRs include Toll-like receptors (TLRs), Nod-like receptors (NLRs) and Rig-like receptors (RLRs). Activation of these receptors results in the alteration of gene expression and the initiation or inhibition of cellular events. These events include induction of pro-inflammatory cytokine and chemokine production, upregulation of co-stimulatory molecules and major histocompatibility complex

(MHC) molecules by antigen-presenting cells (APCs) and subsequent migration to lymph nodes. The activation of PRRs thus couples the innate to the adaptive immune system. Non-signalling PRRs include soluble acute-phase proteins such as C-reactive proteins and lectin and membrane bound scavenger receptors. These function to bind to pathogens making them more susceptible to recognition by the complement system and phagocytosis and subsequent degradation.

While many pathogens are readily eliminated by the innate immune system, some infections are more severe and require the assistance of the adaptive immune system for clearance. However, the adaptive immune system requires a priming period of 4-5 days during which DCs which have identified the invading pathogen via PRR stimulation capture, process and present isolated specific molecular components of the pathogen called antigens to T cells, a type of adaptive immune cell which reside in the many draining lymph nodes located around the body. During these 4-5 days the innate immune system often acts to contain the infection. However, this process can lead to tissue damage by infiltrating innate cells such as neutrophils, often leading to local symptoms such as inflammation and abscesses and/or systemic symptoms such as fever and fatigue.

The adaptive immune system, which is unique to vertebrates, represents a significant milestone in evolution and is executed by two predominant groups of highly specialized lymphocytes called T and B cells. Though individual and distinct subsets of each of these populations have been identified, the true mechanism by which the adaptive immune system can respond uniquely to a seemingly endless number of individual pathogens is through its ability to recognize a vast number of antigens through a variable repertoire of unique surface bound and secreted antigenic receptors.

However, neither B nor T cells carry a complete set of these antigenic receptors. These are generated upon presentation of antigens by DCs, and the consequent expansion of cognate T or B cells, explaining the initial lag between the innate system encountering a pathogen and the initiation of the adaptive immune response for that specific invader. The ability of T and B cells to respond to such a large number of antigenic targets lies in their ability to genetically rearrange the genes encoding their antigen receptors against specific pathogens. Once a high affinity receptor has been selected for, the T or B cells expressing this will rapidly proliferate and expand to neutralize the threat. After resolution of the infection a small population of these T or B cells endures in order to constitute a "memory" function. Upon renewed encounter with the antigen, these memory cells can quickly expand and fight the infection but it is important to mention that neither arm of the immune system acts independently. They are explicitly linked and act together in unison to educate each other and clear pathogens with very high efficiency.

1.2 – Variolation; humans deliberately infect each other!

Although our knowledge of the immune system is vast and ever-growing, little was known about the specifics of the immune system until the beginning of the 1900s. However, historical documents going back to 10,000BC when individuals who had survived the devastating disease smallpox were employed to care for those upon whom the disease fell. It was noticed that once a person survived small pox they could not become infected again. While herbal remedies and elixirs failed to treat the disease, variolation was one outcome of the observation of immunity in certain infected individuals. Variolation involved the inoculation of a non-immune challenged individual with disease tissue from an infected individual such as scar tissue or pus. In the East the dried crusts of smallpox lesions of survivors were ground up and used to inoculate uninfected individuals by insufflation (Figure 1.1). In the West the subcutaneous route of application was preferred.

Pus from smallpox lesions was used to inoculate via a lancet or cut into the skin. This technique was brought from Istanbul in the 18th Century to Europe after the procedure was witnessed in the court of the Ottoman sultan. A trial performed on prisoners (who were granted the kings pardon in exchange for their participation) was a resounding success as all test subjects survived variolation and subsequent smallpox infection [1]. This experiment was repeated in orphaned children and again proved successful, following on to a final test on members of the British royal family, where success led to the general acceptance of the procedure [1]. While the procedure spread rapidly among physicians in Europe, 2-3% of inoculated individuals died from the infection, some contracted smallpox and became a source of an epidemic or contracted another disease such as tuberculosis through the donor of the lesion. However, the fatality rate was still 10 times lower after variolation than naturally contracting smallpox [1]. While variolation would continue for many years in Europe, physicians had unbeknownst initiated the quest for a smallpox vaccine

Figure 1.1 – In response to endemic smallpox, variolation by insufflation of dried crusts from smallpox lesions is used in China by 1000BC.

1.2.1 – Vaccination; the story of cowboys

In the middle of the 1700's Europe was in the midst of a revolution the likes of which had never been seen before. Rapid advances in agriculture, industry, exploration, urbanization and scientific discovery accounted for an explosion in population and wealth. Very little progress however, was made in the field of medicine, with many people still succumbing to infections we today consider either eradicated or predominant only in less developed countries. Indeed despite the rapid development of society as a whole the average life expectancy was a mere 35 years. Furthermore, infant mortality was almost 50% in some countries mostly due to infections. Smallpox still accounted for a large proportion of these deaths. It was one discovery in particular during this era led to the first major public health scheme which subsequently played a key role in the global eradication of an illness almost 300 years later.

Edward Jenner, a physician and scientist from Berkley, Gloucestershire was 8 years old when he was inoculated with smallpox through variolation (Figure 1.2). Jenner once overheard a dairymaid claim "*I shall never have smallpox for I have had cowpox. I shall never have an ugly pockmarked face*" [1]. In fact it was commonly observed that dairymaids often contracted a mild and transient illness called cowpox. This resulted in a disease more uncomfortable than problematic. After a short spell of non-debilitating illness these women were able to return to their everyday lives. Furthermore, the lesions that appeared during this illness did not leave behind the permanent scars which marked those who survived smallpox. Most importantly these women were completely protected against any subsequent smallpox infection. However, it would be many years after concluding his training that Jenner would make the connection that cowpox provides protection from smallpox infection. Having heard the tales told by dairymaids, Jenner concluded that cowpox must protect them against smallpox and furthermore, that it could be transmitted between individuals [1].

On May 14th, 1796 Jenner evaluated his hypothesis by inoculating a young boy, James Phipps with pus from the lesion of a cowpox-infected dairymaid, Sarah Nelmes (Figure 1.2). Phipps experienced a mild fever and some discomfort but no significant infection lasting only a few days. Later Jenner attempted to infect Phipps with material used for variolation from a fresh smallpox lesion and as he hypothesized no disease resulted. Subsequent challenges, even via direct contact with people infected with smallpox proved that Phipps was rendered immune to smallpox by inoculation with cowpox, later identified as the vaccinia virus [1].

Jenner called his discovery "Vaccination" and published his finding in a booklet entitled *An Inquiry into the Causes and Effects of the Variolae Vaccinae, a disease discovered in some of the western counties of England, particularly Gloucestershire and Known by the Name of Cow Pox*. Jenner made no attempt to commercialize his discovery, and although he received many academic and monetary rewards he died of a stroke after withdrawing himself from society after the loss of many family members and friends due to tuberculosis [1]. Although Jenner was not the first to attempt vaccination per se, his work represented the first scientifically verified attempt to control an infectious disease by vaccination. Vaccination with vaccinia virus has since led to the global eradication of small pox in 1979 [2]. This serves as the undeniable proof of Jenner's concept and his epitaph.

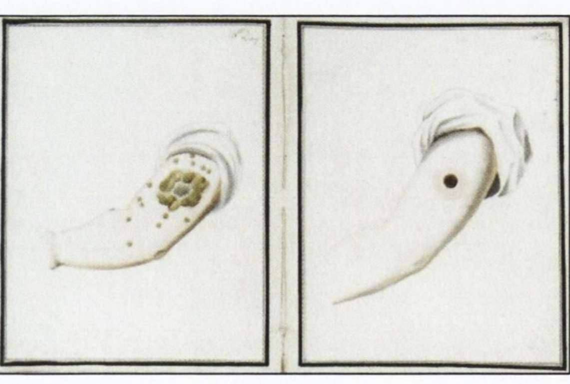
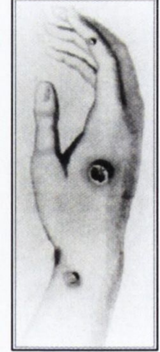



Figure 1.2 – Edward Jenner (left), who took pus from the cowpox lesion of Sarah Nelms (middle) discovered that 16 days after inoculation smallpox leaves a much more pronounced lesions than with cowpox (right).

1.2.2 – Cholera, chickens and a dog bite; Vaccine research begins, a century later.

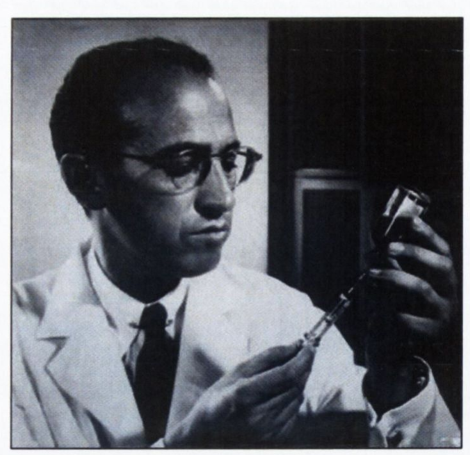
Despite Jenner's success, further vaccines would not be developed until nearly a hundred years later with the recognition that bacteria are one of the casual agents of disease or "germ theory". While conducting work on chicken cholera, Louis Pasteur discovered that *Vibrio cholerae* bacteria, damaged due to age, failed to elicit disease symptoms in chickens. Later he infected these apparently healthy chickens with viable *V. cholerae* and discovered that these did not succumb to nor exhibit the symptoms of the disease. Similar experiments performed on anthrax in cattle revealed that when bacteria were chemically weakened and used to inoculate cattle, these animals were protected against the disease. Pasteur thus showed that one could attenuate a pathogen and use this weakened native organism to induce protection. This was controversially demonstrated by Pasteur in 1885 when he protected a child from rabies after it was attacked by a rabid dog. Despite not being a physician, and placing himself in a position of prosecution, Pasteur inoculated the child with attenuated rabies virus from dried infected rabbit nerves. The boy recovered from the physical injuries of the attack and moreover, did not succumb to rabies. Pasteur was hailed a hero and more importantly was spared from criminal charges. This discovery laid the foundations for the manufacture of preparations which Pasteur called vaccines in honour of Edward Jenner, and the establishment of the first Pasteur institute. Despite two very different approaches, both Pasteur and Jenner had lit a fuse, which would culminate in an explosion of vaccine research that led to an era of vaccine discovery which is still continuing today.

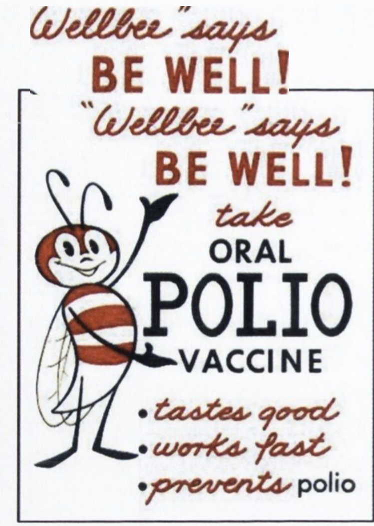
1.2.3 – A vaccine against polio: the blockbuster vaccine

One of the greatest success stories of modern vaccine discovery was the development of the polio vaccine by Jonas Salk (Figure 1.3). Poliomyelitis is a serious disease that often resulted in paralysis and death. The causative agent of this disease, the polio virus, is usually spread by

the faecal-oral route. Victims of polio would often have to be placed in an "iron lung" to assist with their breathing and often suffered acute limb paralysis and deformation in later life due to atrophy. This disease was especially dreaded in the 20th Century until Jonas Salk's vaccine began to turn the tide on the disease. Salk's vaccine consisted of an inactivated polio vaccine (IPV), containing three virus strains grown in monkey kidney cells and inactivated with formalin. This injectable vaccine reduced the number of cases of polio in the United States from 35,000 in 1953 to 161 cases in 1961, a mere 6 years after the launch of Salk's vaccine in 1955.

Concurrently with Salk's efforts, researchers were also conducting experiments on an oral polio vaccine (OPV). A group led by Albert Sabin (Figure 1.3) developed a live-attenuated polio virus by passaging the virus several times through human cell lines at various suboptimal conditions to introduce genetic mutations that would reduce viral virulence while maintaining its immunogenicity. These efforts culminated in large scale clinical trials conducted within the Soviet Union in the 1950s which proved the safety and effectiveness of the OPV concept. These results led to the licensing of Sabin's OPV in 1962. The National Institute of Health launched a large scale OPV campaign in the United States in 1963, which largely replaced Salk's IPV vaccine due to the lower cost and ease of administration. In fact 20 doses of OPV can be applied to sugar cubes and administrated to children in the time it takes to load and administer a single dose of the IPV, and without the discomfort of the injection. This was the first large scale demonstration of the benefits of oral vaccines versus traditional injectable vaccines. So effective was the OPV campaign (Figure 1.3) between 1963 and 1955 that it further reduced the number of polio cases in the United States from the already dwindling numbers due to the IPV.





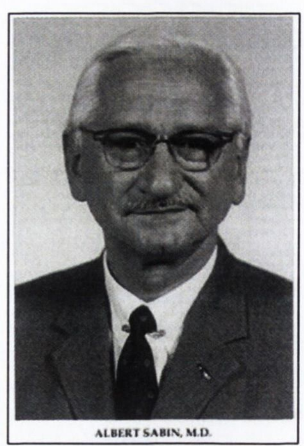


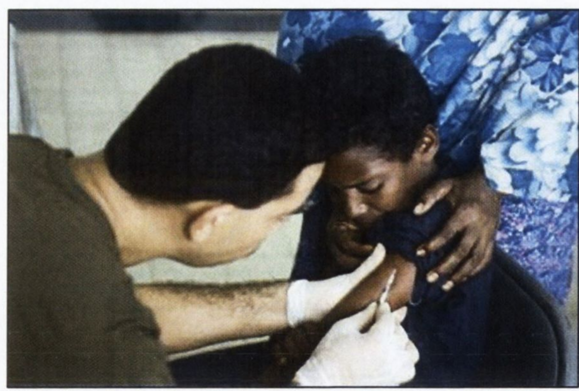
Figure 1.3 – The founding fathers of the inactivated polio vaccine; Jonas Salk (left) and of the oral polio vaccine; Albert Sabin (right). Following the discovery of both the IPV and OPV mass vaccination campaigns were launched, including "Wellbee" (ca 1963) for the OPV (middle).

Despite its success in the early 1960's, OPV has been largely abandoned by western health systems as part of their vaccination programs due to two concerns. It has been reported that polio virus can be detected in the stool of vaccinated individuals. This is likely due to shedding of the virus after non-disease causing colonization of the intestinal epithelium. However, of greater concern, several cases of polio arising after vaccination began to surface. This was due to the OPV virus reverting to its virulent form and causing latrogenic (vaccine-induced) polio. Despite being a rare event, cases of vaccine-associated paralytic poliomyelitis (VAPP) are reported [3]. Rates of VAPP vary greatly in different global regions, but it is agreed that it can occur in approximately 1 in 750,000 OPV recipients. Taken together this could lead to the spread of reactivated polio virus in faecal matter in areas that had been largely eradicated of polio. Despite its discontinuation in 2000 in the USA, the OPV continues to be used in developing nations were high levels of wild type polio continue to be detected in the environment. If these levels are reduced over time a move from the OPV to the IPV is recommended as the IPV does not contain a virus that can revert to its virulent form and be transmitted via faeces.

1.3 – Mucosal Vaccines; the next stage in vaccine development?

The effectiveness of vaccines has been so profound that the life expectancy between the 1700s and 2000s has more than doubled. However, this usually relates to developed countries in North America, Southeast Asia and Northwest Europe. While infectious diseases no longer account for the majority of deaths in these countries, they remain a major cause of mortality in developing countries. Interestingly, the vast majority of licenced vaccines are administered parenterally while 70% of pathogens enter the body via a mucosal tissue such as the lung or gastrointestinal tract (GIT) [4]. Parenterally administered vaccines however, do not elicit protective immune responses at these mucosal sites. These vaccines protect against the systemic manifestation of the disease, predominantly via immunoglobulin (Ig) G in the serum (as is the case for polio) but do not prevent initial attachment at or entry through the mucosa by the pathogen.

Mucosal vaccines are regarded as an advantageous alternative to parental vaccines from a production, economic and regulatory perspective [5, 6]. In terms of production, antigen and adjuvant formulations for oral administration generally require less purification from bacterial by- products such as endotoxin, than parentally administrated ones as the gut is already populated by microbes which produce a milieu of endogenous by-products. Better compliance and a reduced risk of adverse reactions to a vaccine are some of the safety benefits of the mucosal route. Economically, mucosal vaccines are also suited more to mass-vaccination schemes as no needles are required reducing the risk of disease transmission through contaminated equipment and the need for highly trained medical personnel to operate these is also reduced (Figure 1.4) [7, 8]. Pandemics such as influenza and epidemics resulting from disease outbreaks during natural disasters and conflicts could be quickly contained and even prevented using mucosal vaccination [9-11].



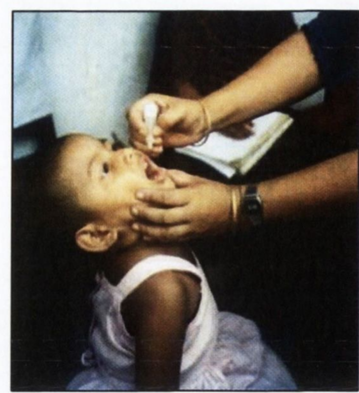


Figure 1.4 – Although injectable polio vaccines (left) elicit outstanding protection against poliomyelitis, the practical and economic advantages conferred by oral polio vaccines (right) have led to their preferred use in developing countries.

Despite sustained efforts to develop mucosal vaccines, only few have been licensed. Many vaccines have been effectively evaluated in animal models but have failed in clinical trials. However, live-attenuated vaccines against rotavirus, influenza virus and the causative agent of typhoid fever, *Salmonella enterica* have all been successfully used despite a safety and stability concern with live-attenuated vaccines. Two killed whole cell killed (WCK) vaccines against cholera, Dukoral® and Shanchol™ have also been licensed and are a proof of concept that non-living mucosal vaccines are feasible [12]. One drawback with WCK and subunit vaccines is that these often suffer from poor immunogenicity and require the inclusion of adjuvants. These are just a few of the challenges that mucosal vaccination faces. Despite these challenges, the advantages of mucosal vaccination often out weight the challenges and thus there is renewed interest in the development of mucosal vaccines in order to establish a protective immune response at the site of pathogen entry. Before one can begin to develop a candidate mucosal vaccine one must consider the complexity of the mucosal immune system which varies greatly from the systemic system and between mucosal routes.

1.4 – Routes of mucosal vaccine administration

Several delivery routes for mucosal vaccines exist (Figure 1.6). Nasal, oral and sublingual represent three of the most accessible routes, while rectal and vaginal routes have been explored in animal models. However, cultural considerations and taboos may limit the real world application of the latter two mucosal routes. Initially the oral route was most intensively explored as this was the traditional route of non-injectable drug delivery in humans. Recently increased interest in nasal and sublingual for protective and immunotherapeutic strategies respectively has demonstrated successful results leading to the licensing of several such products [13].

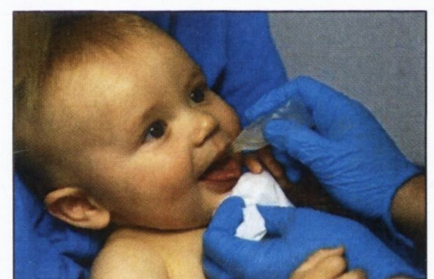
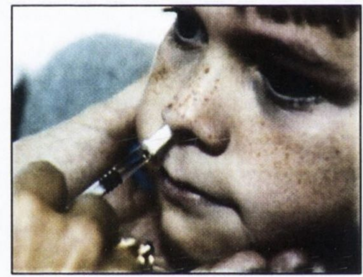
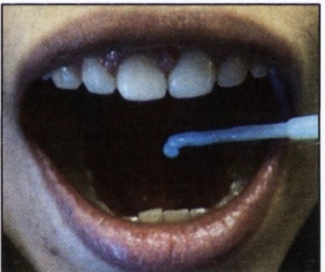




Figure 1.5 – Three predominantly utilized routes of mucosal vaccination include oral (left), intranasal (middle) and sublingual (right).

1.4.1 – Oral Vaccines

Oral vaccination presents the most desirable and yet most challenging route for mucosal immunization (Figure 1.5). Challenges include the acidic and harsh enzymatic environment of the stomach and upper portions of the small intestine, the predisposition towards tolerance of the gut immune system and the plethora of food, external environmental and commensal antigens that will compete with the vaccine possibly causing dilution of the vaccine

formulation. Despite these challenges, numerous groups have reported successful results in animal studies; however, reproducing these in human subjects has proved to be a challenge [13-15]. This has promoted the search for more sophisticated formulations that address the challenges of oral vaccination. Although efforts to develop live-attenuated vaccine formulations continue, it is generally regarded as more advantageous to develop killed or subunit vaccines with potent mucosal adjuvants as they generally present improved safety profiles and storage stability [5, 16]. Although it is generally well accepted that particle-based vaccine formulations have higher immunogenicity at mucosal sites, such a formulation has yet to be licensed for human use [17, 18]. Despite these challenges, licensed oral vaccines do exist, which provide mucosal protection, demonstrating the effectiveness of this route. The issue with emulating the relative success of these vaccines for the generation of new vaccines and the hurdles to improving the existing ones are the incomplete understanding of how mucosal protection by oral vaccines in humans is elicited. There are also significant differences between the types of protection required for different mucosal pathogens so tailored vaccine formulations are required. Increased understanding of the mode of protective immunity elicited by currently licensed oral vaccines would significantly aid progress in the development of new oral vaccines and improving existing ones [16].

1.4.2 – Intranasal Vaccines

While oral vaccines are utilized for their ability to induce protective immunity in the GIT, intranasal vaccination is a desirable method for generating immune responses in the nasal cavity and respiratory tract. Intranasal vaccination stimulates the immune system of the nasopharynx, which not only stimulates immunity in nasal and respiratory tissues [19], but may promote immune responses in the mucosa of the GIT and genital tracts [20]. The only licensed intranasal vaccines are live-attenuated seasonal influenza strains [9] (e.g. FluMist®) which also provide cross-protection against other influenza strains [21]. Intranasal vaccination

is an attractive route as it requires a lower antigen and adjuvant dose than oral vaccination. However intranasal vaccination may be more costly that oral vaccination and less practical as delivery devices and trained staff still need to be present for the administration. The presence of olfactory nerves in the nasopharynx is also a safety concern for certain formulations (section 1.11.1).

1.4.3 – Sublingual vaccination

Interest in the sublingual route of administration (i.e. under the tongue) has been gathering momentum [22]. Interestingly the sublingual route has the ability to induce strong mucosal and systemic responses at distant mucosal sites such as the genital tract [23]. Sublingual immunisation can also be used as an immunotherapeutic tool to generate tolerogenic responses against allergy causing antigens. In contrast to intranasal vaccination, the sublingual route avoids contact with nerves of the central nervous system, eliminating that safety concern [22].

1.5 – Mucosal Tissue, slippery when wet.

In order to develop improved mucosal vaccines it is important to understand the mucosal immune system. Many regard the skin as the largest organ in the body, which may be true if the criteria were based on apparent size. However if one was to use surface area as a criterion, the combined surface area of the GIT, respiratory tract and genitourinary tract dwarfs the surface area of the skin by approximately 200 times. The total surface area occupied by the adult human mucosa is 400m^2, equivalent to almost 1 and a half tennis courts and most of this is occupied by the small intestine [24]. Although each surface has evolved to fulfil a specific physiological role, in the GIT; nutrient uptake and waste excretion, in the respiratory tract; gaseous exchange and in the genitourinary tract; waste excretion and reproduction, certain features of these organs are conserved between all three. All mucosal organs, especially the

GIT and respiratory tract, are constantly exposed to the external environment, which contains a huge number of different antigens both harmful and harmless. The constant pressure of exposure to environmental antigens led to the evolution of a highly specific system of immunological surveillance where mucosal APCs relay information about the external environment to mucosal effector cells via a network of cells, cytokines and chemokines. This large and varied network of mucosal tissues, lymphoid and non-lymphoid cells, messenger and effector molecules synchronizes and integrates in order to confer protection against mucosal pathogens and maintain homeostasis [25]. It is these networks within the mucosa, where the antigen-specific immune responses are coordinated and executed. In order to further the development of efficacious mucosal vaccines, these immune mechanisms must be extensively characterized and understood.

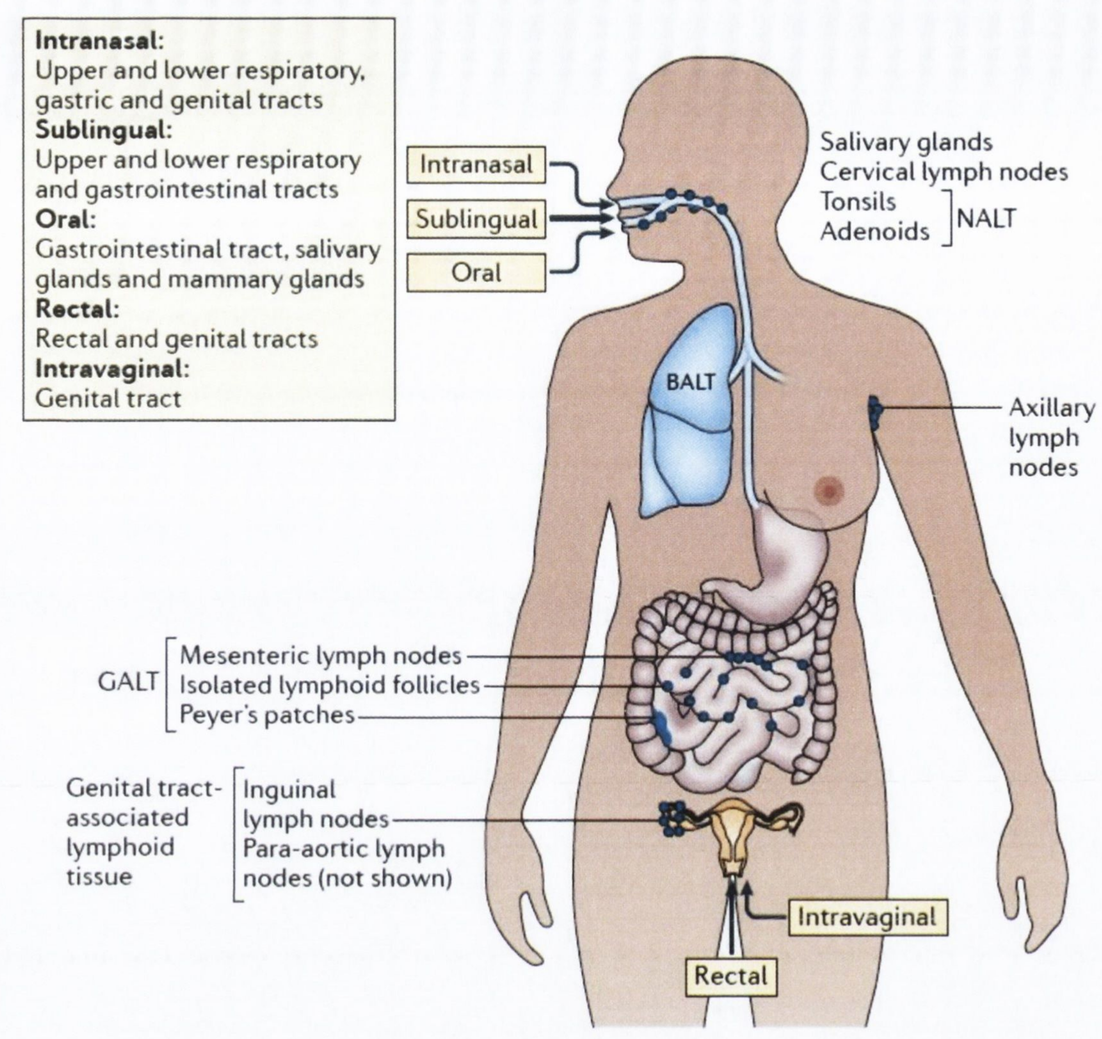


Figure 1.6 – Routes of mucosal immunization and the anatomical locations of mucosal effector sites. The mucosal-associated lymphoid tissue (MALT) is a general term used to describe the sub-anatomical compartments of mucosal organs where immune responses are initiated. In the nasal cavity this is the nasopharynx-associated lymphoid tissue (NALT), respiratory tract the bronchus-associated lymphoid tissue (BALT), in the gut the gut-associated lymphoid tissue (GALT) and the genital tract-associated lymphoid tissue. In order to elicit an immunological response in one tissue it is important to target the correct MALT to ensure a favourable outcome. So vaccination routes can also elicit responses in distant MALT tissues. Oral (intra-gastric) vaccination effectively stimulates antibody-mediated immunity in the gastro-intestinal tract (GIT) and in secretions from the mammary glands. Intranasal vaccination has been shown to effectively stimulate both antibody and cellular immune responses in the respiratory, GIT and genital tracts while rectal immunization induces strong responses in the colon, rectum and genital tract. Intra-vaginal vaccination is effective at generating strong antibody and T cell responses in the genital tract. (Taken from Lycke, N. et al, 2012)

1.6 – Mucosa-associated lymphoid Tissue

The aim of vaccination is to induce a memory response that upon challenge with the target pathogen will induce a response sufficiently robust to clear the infection without major damage to the host. However, to accomplish this one must first consider the sites at which the vaccine will be encountered. The sites at which antigen-specific immune responses are initiated in mucosal tissues are known as the mucosa-associated lymphoid tissue (MALT) structures [26-28] (Figure 1.6). This tissue is characterized by a large number of lymphoid follicles, which are distributed across the tissue (Figure 1.9). In the GIT the MALT, or gut associated lymphoid tissue (GALT) (Figure 1.9), is primarily composed of lymphoid organs known as Peyer's Patches (PPs) and isolated lymphoid follicles (ILFs), while the nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) is the primary MALT found in the nasal and respiratory tract. In contrast the genitourinary tract does not contain MALT and thus utilizes a very different system to prime the adaptive immune system [29]. There are many differences between the immune system present in peripheral lymph nodes and mucosal tissues and even between the different mucosal tissues. Differences in the type of cells and their respective contribution to the mucosal immune responses can be used to separate mucosal surfaces into either type I or type II mucosae [30]. The surfaces of the lung, GIT and upper portions of the female reproductive tract contain type I mucosae while the mouth, cornea and lower female reproductive tract contain type II mucosae. The two mucosae can be distinguished by the type of epithelium present, the mechanism by which IgA and IgG is transported (active or passive), the presence (type I) or absence (type II) of MALT and the composition of local immune cells. Since type I mucosae comprise the primary mucosal tissue found in the GIT, and thus the target of oral vaccines, these will be the primary focus of this introduction. Mucosal vaccine-induced immune responses are initiated in MALT structures, which are regarded as the major

inductive sites of mucosal immunology [26-28]. Type I mucosal tissue contains anatomically distinguishable sites where responses are initiated (the organized lymphoid tissue) and where effector responses are exerted (diffuse lymphoid tissue). Diffuse lymphoid tissue contain a majority population of lymphocytes which reside as either intraepithelial lymphocytes in the mucosal epithelial barrier or in the large network of connective tissue underlying the mucosal barrier known as the lamina propria (LP). The individual structures found in the MALT, specifically those in the GALT will now be revived to highlight their specific involvement in the initiation and execution of gut immune responses as these pertain most importantly to oral vaccination.

1.6.1 – Antigen uptake in the gut

Multiple systems exist in the intestine to sample, process, deliver and present antigens to lymphocytes. Antigen sampling and presentation can occur via different mucosal DC subsets [31, 32], microfold (M) cells [33, 34] and mucus secreting goblet cells [35]. M cells have specifically evolved to take up antigen, making them accessible targets for antigen delivery (Figure 1.7). Specialized at the capture, uptake and transport of antigens, M cells are phenotypically very different from the other mucosal epithelial cells whose phenotype is that of an absorptive enterocyte, specialized for nutrient uptake. In contrast to the epithelium, M cells lack the organized brush boarder and cilia present on enterocytes [36]. The lack of a brush boarder allows the M cells to effectively take up particulate antigens, bacteria and viruses, and by transcytosis delivery these to underlying lymphocytes, via direct contact in the basolateral pocket directly below the M cell [37]. Antigens are delivered directly either to follicular B cells or mucosal DCs which in turn present the antigen to T and B cells in the MALT or migrate to the mesenteric lymph nodes. However, despite M cell targeting showing promise for enhancing oral vaccine efficacy, one must also consider the complicated milieu of cells, interactions and effector molecules that exist in the intestinal mucosa. An effective oral

vaccine should be constructed in such a manner that the desirable pathways governing potent mucosal immunity to that pathogen are activated.

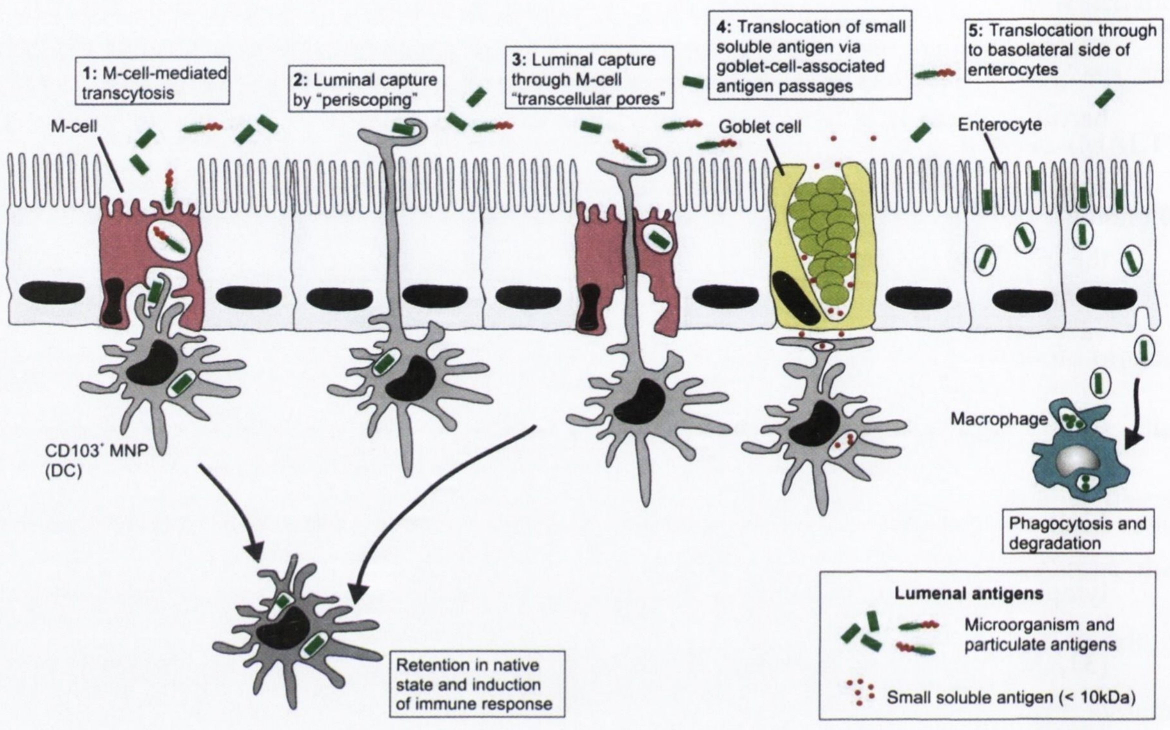


Figure 1.7 – Potential route of luminal antigen uptake in the intestine. 1) Microorganisms and macromolecular antigens are taken up by microfold (M) cells and passed to the basal side of the intestinal epithelial barrier to dendritic cells (DCs) by transcytosis. 2) Mononuclear phagocytes (MNPs) can extend dendrites through the tight junctions between intestinal epithelial cells (IECs) to directly sample luminal contents. 3) MNPs extend dendrites through "transcellular pores" in M cells and sample luminal contents directly. 4) Mucin-secreting goblet cells function not only to replenish intestinal mucus layers, but can also translocate and present small soluble antigens to MNPs on the basal side of the intestinal barrier. 5) Follicle-associated epithelial enterocytes can endocytose certain gut antigens in the lumen and exocytose these in late endosomes into the extracellular space in the subepithelial dome (SED) where they are acquired by MNPs. (Taken from Mabbott, N.A. et al, 2013)

1.6.2 – Peyer 's Patches

To induce effective mucosal immune responses in the gut it is desirable to direct a vaccine towards the main sites of mucosal immune activation in the gut, namely the PPs (Figure 1.8). PPs are the primary inductive sites of intestinal immune responses and reside under the intestinal epithelium. These large structures are built on a stromal cell (SC) scaffold, rich in B cell follicles which are separated by APCs such as DCs and lymphocytes including T cells (Figure 1.8). It is also in these tissues that isotype switching of naïve IgM+ B cells to mature IgA+ plasma cells occurs [38, 39]. The majority of activated lymphocytes in PP reside in germinal centres (GCs). These specialized micro-environments have evolved to promote interactions between B cells and resident SC APCs called follicular dendritic cells (FDCs). FDCs are assisted by a specialized lymphocyte cell subset called follicular helper T cells (TFH) [40]. Mice deficient in T cells suffer from an absence of GCs in their PPs and mesenteric lymph nodes (MLNs) [41-43]. The requirement for TFH cells in the induction and activation of GCs [40] can be attributed to their unique positioning in relation to FDCs in the GC, determined by the differential expression of chemokine receptors on the TFH cells [44]. TFH cells also express molecules that are classically associated with T helper functions such as CD40 ligand (CD40L) and interleukin IL-21 [40]. Together the FDCs and TFH cells efficiently supply conditions that are conducive to efficient class switching of naïve B cells to IgA secreting plasma cells, antigen presentation and T-B cell interactions. Interestingly, B cells can also form GCs independent of B cell receptor (BCR) stimulation via direct sensing of bacterial components by TLRs on B cells [41]. This model is supported by a finding that T cell receptor (TCR) stimulation of B cells elicits enhanced IgA responses via a myeloid differentiation primary response gene 88 (MyD88)-dependent signalling cascade [45].

While the mechanisms behind the predisposition of class switching from IgM+ to IgA+ B cells in GCs in the PPs remains elusive, evidence suggests that resident DCs play a large role

in establishing this microenvironment. This preference of IgA class switching may be due in part to the location of the PP DCs in the sub-epithelial dome (SED), where they can sample antigens and bacteria directly from the intestinal lumen, which are bound to or coated in secretory IgA (SIgA) [46], despite M cells being previously considered the primary site of antigen entry into PPs [4]. PP DCs do not exclusively participate in local PP IgA induction; rather they can migrate to the MLNs where they can initiate preferential class switch recombination (CSR) to IgA in MLN B cells [47]. T cells present in the PPs are also educated by gut DCs to produce cytokines that are conducive to class switching of IgM+ B cells to IgA [48-50].

In the presence of a normal microflora, GCs are constituently present in PPs and in the MLNs. It is in these environments that most of the gut IgA producing plasma cell precursors are generated [41, 51]. Indeed most B cells present in GCs are activated, characterized by the expression of activation-induced cystidine deaminase (AID). AID has been shown to be critical for CSR and somatic hypermutation (SHM) to occur, both of which are key steps to generating a large, varied and dynamic population of antigen-specific plasma cells [43, 52]. The IgA response generated in GCs is also antigen-specific and not simply a polyclonal B cell response to TLR stimulation.

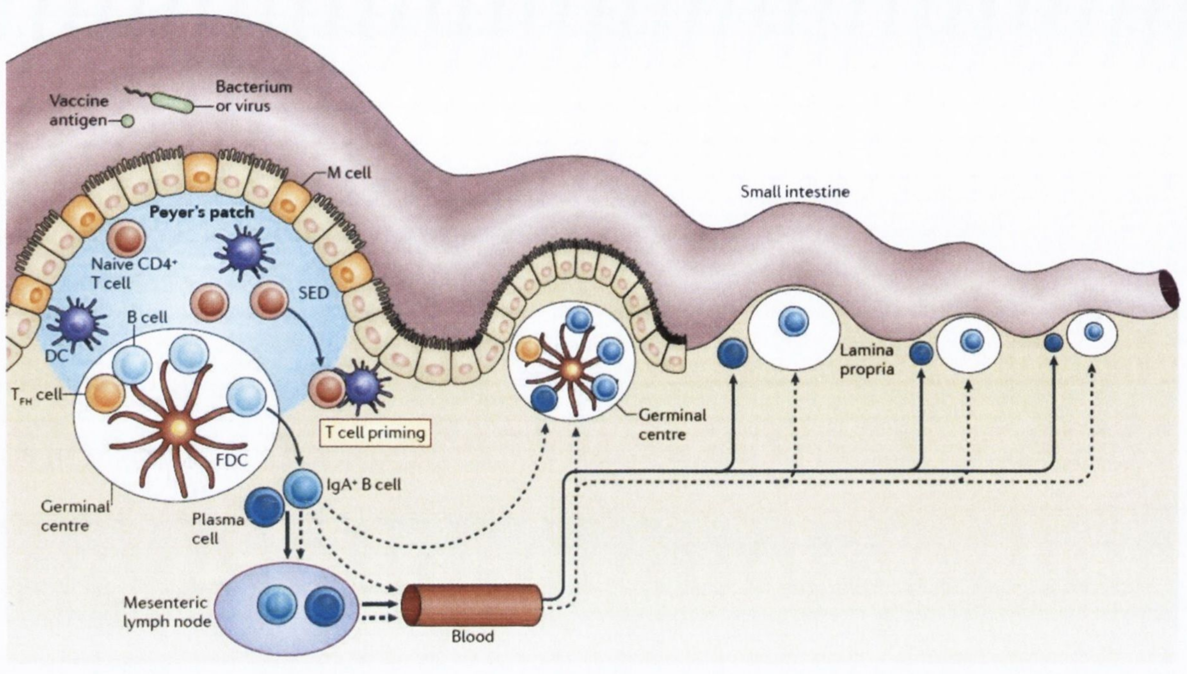


Figure 1.8 – Mucosal vaccine elicited IgA induction in the Peyer's patches of the small intestine. Most antigens in the gut are taken up by specialized microfold (M) cells which overly the Peyer's patches (PPs) and lack the mucus layer and brush border of the conventional intestinal epithelium. Dendritic cells (DCs) resident in the sub-epithelial dome (SED) take up antigens from the basal side of M cells, process and present these to naïve T cells in the interfollicular space. Follicular helper T cells (TFH) cells interact with cognate B cells at the border of B cell follicles. Next a germinal centre forms as Tfh cells colocalize with B cells in the presence of fDCs. Inside the germinal centre, antigen-specific B cells undergo class-switching and somatic hypermutation to generate high affinity IgA. This process generates both long-lived IgA+ plasma cells and memory B cells within the germinal centres. These then egresses from the PPs via the efferent lymph and migrate to the mesenteric lymph nodes. From the mesenteric lymph nodes (MLNs) plasma cells home to the bone marrow via the blood and on to other effector sites in the lamina propria (LP) of the GIT. The antibody response following oral vaccination is highly synchronized and oligoclonal. The antigen-specific IgA+ B cells and plasma cells in both the GALT and LP are clonally related, which may suggest that multiple PP are engaged in the establishment of mucosal immunity in the gut. This immunity occurs when existing germinal centres in numerous PPs are actively expanding and selecting high-affinity antigen-specific IgA+ B cells, which may explain why multiple rounds of oral vaccination are required to elicit protection by driving high affinity IgA responses in the LP. (Taken from Lycke, N. et al, 2012)

1.6.3 – Isolated Lymphoid Follicles

Smaller variants of PPs are scattered along the length of the intestine and are known as ILFs (Figure 1.9). While similar to peripheral lymph nodes, this organized lymphoid tissue contains a higher proportion of B cells to T cells compared to its systemic equivalent. Covering ILFs is the intestinal epithelium which contains M cells. These are capable of taking up pathogens from the intestinal lumen such as *Salmonella typhimurium*, which leads to the formation of GCs in the mature ILFs (Figure 1.11) [53-55]. Interestingly, it was found that the presence or absence of GCs in ILFs did not influence the active class switching of naïve B cells to active IgA secreting B cells. More so this study also found that unlike PPs, the induction of AID and generation of IgA secreting plasma cell precursors is T cell-independent in the ILFs [56]. ILFs could be considered a unique means of generating GC and T cell-independent activation of B cells and their class switching into IgA secreting plasma cells in the gut, in order to contribute to the large daily turnover of IgA. It seems this is achieved by the DCs in the ILFs themselves, which are sufficient to activate B cells [56]. In contrast to splenic and peripheral DCs, gut macrophage (Mφ)-DCs are known to secrete large amounts of TNF-α following bacterial stimulation. TNF-α and inducible nitric oxide synthase (iNOS) producing DCs are required to elicit gut IgA production [57]. Furthermore, these gut-specific Mφ-DCs are capable of activating large amounts of transforming growth factor beta (TGF-β) 1 [56]. Both Mφ-DCs and SCs also secrete two additional factors, B cell activating factor of the tumor-necrosis factor family (BAFF) and a proliferation-inducing ligand (APRIL), both of which have been shown to enhance CSR to IgA independently of T cells [57-59]. Thus T cell independent B cell activation may be achieved either directly by LPS sensing on B cells or after antigen presentation by TNF-α-expressing Mφ-DCs. Preferential CSR to IgA may be achieved through the secretion of TGF-β1, BAFF and APRIL. Activated IgA+ B cells in the ILFs may then undergo further differentiation into IgA plasma cells in the

LP under the control of IL-6, IL-10, BAFF and APRIL secreted by the immune cells resident in that compartment of the GALT.

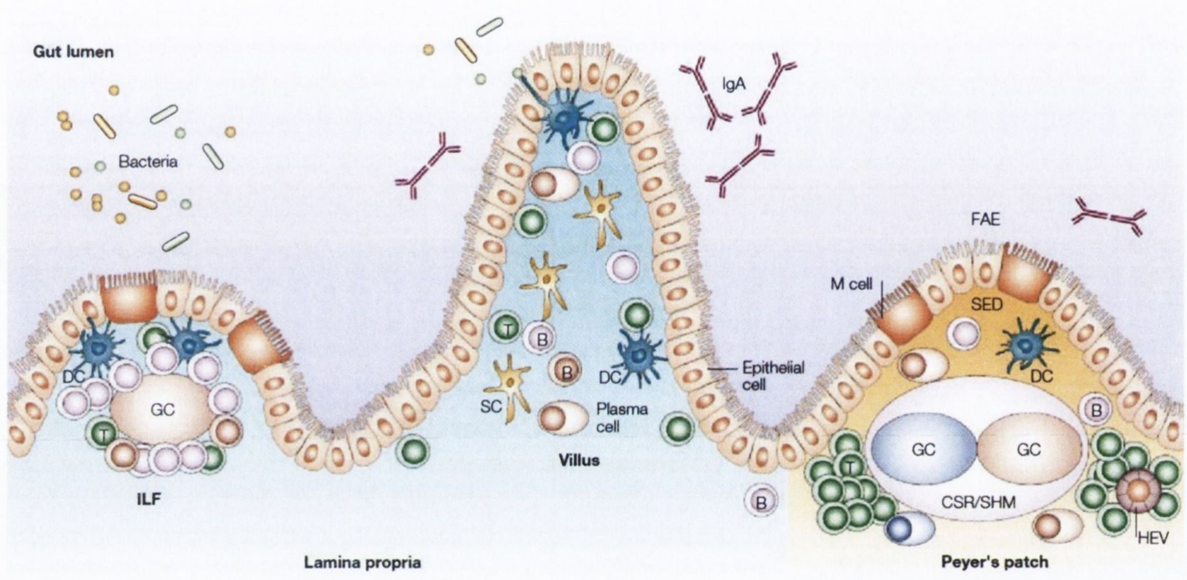


Figure 1.9 – The Gut-associated lymphoid tissue. The induction sites for mucosal immune responses are the Peyer's patches (PPs), isolated lymphoid follicles (ILFs) and lamina propria (LP). PPs and ILFs are composed of the specialised follicle-associated epithelium (FAE) which contains M cells which overlay the sub-epithelial dome (SED), a region rich with DCs and B-cell follicles containing germinal centres (GCs). Here within GCs follicular B cells undergo class-switch recombination (CSR) and somatic hyper mutation (SHM). B cells are also capable of migration through high endothelial venules (HEVs), located in the interfollicular regions of the PPs which are rich in T cells. The diffuse tissue of the LP contains many IgA+ plasma, T and B cells, macrophages, DCs and stromal cells (SCs). LP-DCs can extend dendrites into the intestinal lumen to sample antigen which can be directly presented to T and B cells, thus initiating IgA-class switching and differentiation in the LP. IgA in the LP can be transported across the intestinal epithelial cells (IECs) into the lumen were it serves protective and homeostatic roles. IgA+B cells and plasma cells are shown in red, IgM+ cells in pink and T cells in green. (Taken from Fagarasan, S. et al, 2003).

1.6.4 – Lamina Propria

Although both PPs and ILFs represent significant sites for the generation of IgA plasma cells, it has been shown that class switching of IgM+ to IgA+ B cells can occur in the LP in the absence of PPs and ILFs [55, 60] (Figure 1.10). Furthermore, in situ studies have shown that these B cells are activated with demonstrated AID expression and CSR occurring [61]. Activation of these LP B cells is likely to occur either via antigen uptake from M cells found covering intestinal villi [33, 62] or via LP-DCs which have the ability to extend dendrites through tight junctions in the epithelium to sample antigens directly from the gut lumen (Figure 1.7) [31, 32]. Goblet cells located between IELs have also been shown to be capable of taking up antigen and presenting it to LP-DCs in the lamina propria (Figure 1.7) [35]. Once activated these LP-DCs may be sufficient to induce CSR in LP B cells via a BAFF receptor pathway in the absence of T cells. Interactions between bacteria, gut epithelial cells and DCs have been reported to induce T cell and CD40- independent CSR in B cells via BAFF and APRIL [59]. This response was potentiated through the production of APRIL by the epithelium as a result of TLR stimulation [58]. Epithelial cells further augment CSR via production of thymic stromal lymphopietin which acts on local TNF-α and iNOS-producing DCs to enhance IL-10 and APRIL production [57, 58]. This indicates that T cell-independent IgA responses can be initiated and elicited from the LP by intestinal antigens and bacteria.

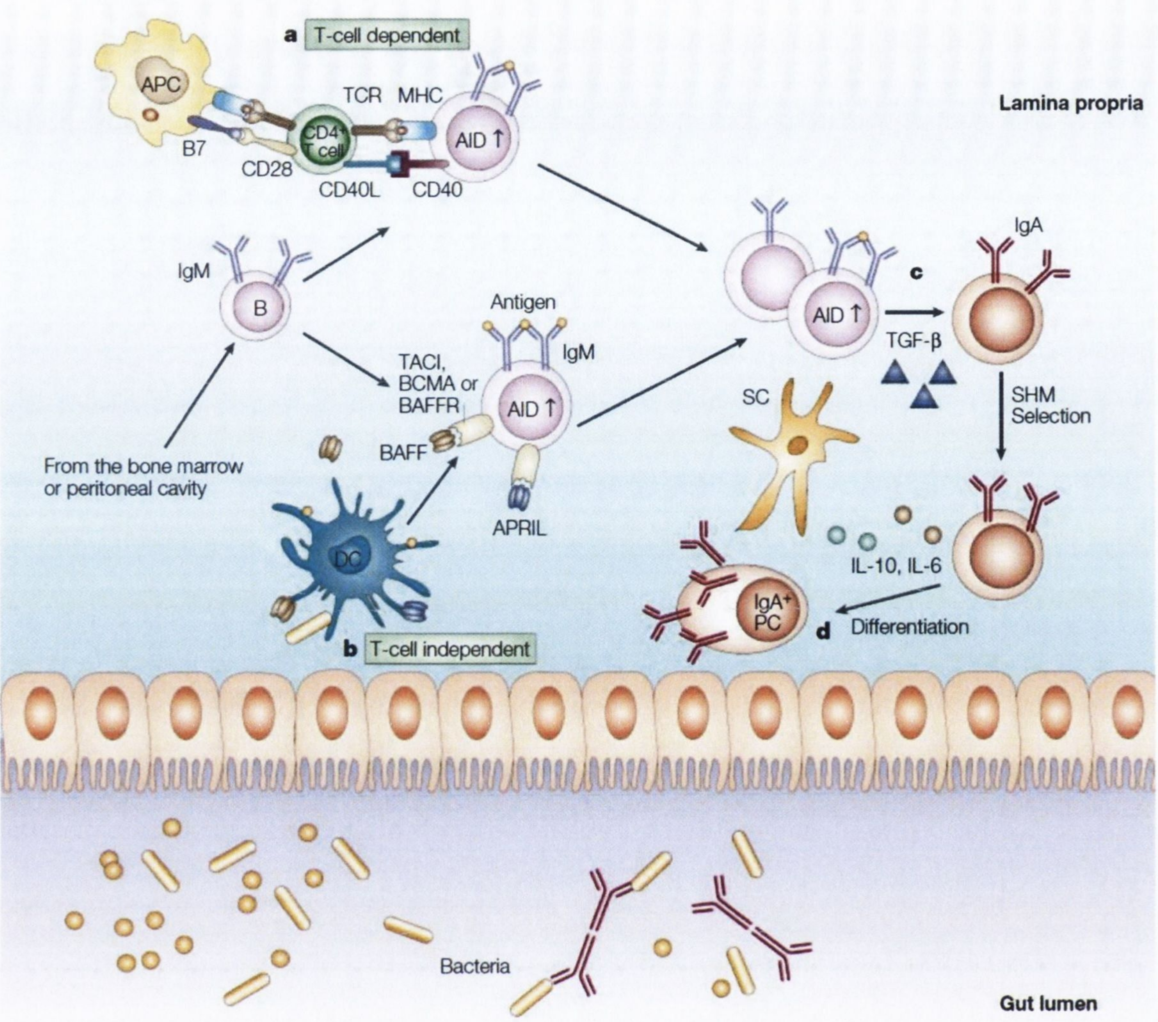


Figure 1.10 – Pathways for the induction of IgA responses in the lamina propria. Naïve IgM+ B cells home to the lamina propria from the bone marrow or peritoneal cavity where they are (a) activated upon encountering antigens and CD40 stimulation via CD40 Ligand (CD40L) and cognate T cell receptor (TCR)-expressing T cells or (b) by antigens presented directly by LP- DCs or other polyclonal stimuli. (a) T cell-derived cytokines and (b) B cell activating factor of the tumor-necrosis factor family (BAFF) and or a proliferation-inducing ligand (APRIL) from antigen presenting cells (APCs) can also augment B cell responses. (b) T-cell independently activated B cells up-regulate activation-induced cystidine deaminase (AID) which is required for class switch recombination (CSR) and somatic hyper mutation (SHM). (c) Cytokines secreted by LP-SCs such as IL-6, IL-10 and TGF-β not only contribute to an environment that favours IgA class switching, but also (d) differentiation of antigen-selected cells into IgA+ plasma cells. (Taken from Fagarasan, S. et al, 2003).

1.6.5 – Gut mucosal homing of T and B cells

The preferential generation of IgA+ B cells in the PPs also imprints these with specific gut homing factors [49, 55]. Mucosal IgA+ B cells, plasma cells and T cells express α4β7 integrin after activation in the GALT. This imprinting occurs when lymphocytes are antigen-specifically activated by mucosal DCs (usually CD103+ DCs) in GALT sites. This imprinting function of DCs is dependent on the vitamin A metabolite retinoic acid (RA) [63-65]. Specific targeting of CD103+ DCs was shown to stimulate effective mucosal homing of activated T cells [66]. PP DCs produce RA which induces the up-regulation of the molecules CCR9 and integrin α4β7, both gut-homing receptors on T and B cells [49, 67]. The egress of IgA+ plasma cells from PPs is mediated at least in part by the type-1 sphingosine 1-phosphate (S1P) receptor [68]. Thus B cells exit the PPs and migrate to the MLNs via the thoracic duct and blood and finally migrate back to the gut. α4β7 allows the cells to specifically attach to high endothelial venule cells by binding to the mucosal addressin cell adhesion molecule-1 (MADCAM-1) located on the epithelial cells of the post capillary venules in the gut LP and so guides the B cells toward gut effector sites [55]. MADCAM-1 is only expressed in the gut mucosa [69, 70]. Lymphocytes expressing CCR9 home to epithelial cells in the intestine that secrete the CC-chemokine ligand (CCL) 25. CCR10 is a chemokine receptor expressed by activated IgA+ B cells and plasma cells which responds to the ligand CCL28 is expressed by epithelial cells in both the large intestine and genital tract, which may account for the ability of NALT to induce effector responses at both these location after intranasal vaccination [71]. The expression of either CCR9 or CCR10 allows imprinted lymphocytes to therefore home to either the small or large intestine where endothelial cells are producing either CCL25 and CCL28 respectively adding further gut homing specificity [72] [73].

1.6.6 – Effector responses in the intestine

When effector responses are generated at GALT sites the resulting T and B cells home to the LP or between epithelial cells; intra-epithelial lymphocytes (IELs) in the gut barrier [74-76]. The desired outcome of mucosal vaccination is the reinforcement of mucosal barriers through SIgA antibodies [28, 77] and effector T cells which enhance mucosal barrier functions [74, 78]. Other local responses to mucosal vaccination include the augmented secretion of host factors such as anti-microbial peptides, defensins, cytokines and chemokines in response to the pathogen [79, 80].

Mucosal vaccination can elicit protection against pathogens at both local and distant mucosal surfaces as well as systemic immune responses [28, 77], inducing both humoral and cell-mediated immunity [27, 74]. The molecules or receptors required to home to mucosal tissues are only acquired in the mucosal draining lymph nodes by interactions with specialized mucosal APCs which migrate to the nodes upon activation after antigen encounter [75-77]. This is thought to be the reason why vaccine administration at sites other than the mucosa is such poor inducer of mucosal immunity. Efforts to enhance antigen uptake by mucosal APCs, the development of mucosal adjuvants to boost protective immune responses and new delivery methods are all being addressed to enhance the protective efficacy of novel mucosal vaccines at the site of pathogen entry.

1.6.7 – Gut T cells and their relationship with gut IgA responses

T cells may preferentially migrate to the intestine upon activation at mucosal distant sites. These may then constitute a local reservoir of memory cells at the frontline of pathogen entry [74]. The relative proportion of B to T cells in mucosal tissue is also much higher than in peripheral tissues, possibly reflecting the difference in the importance of each cell type to the preferential immune response elicited at each site. This may account for the higher

dependence on the T cell-independent B cell responses by B-1 cells in mucosal tissues to bacterial polymeric antigens [81]. The importance of this population of B cells is also seen during neonatal development, when the T cell arm of the adaptive immune system is still developing. Despite the importance of a T cell-independent activation of B cells in the mucosa, T cell-dependent responses still play a key role in the initiation of protective B cell driven responses (Figure 1.11).

1.7 – Antibodies

Antibodies are the secreted form of the BCR and constitute a diverse family of proteins contain Ig domains. They can be secreted as monomers, dimers, and multimers. Antibody molecules can be divided into two regions, each which serves a distinct immunological function. The constant region, mediates the effector function of the antibody molecule while the variable region contains the antigen-binding sites. As the name suggests the variable region is the site at which the huge repertoire of different binding sites are generated and it is this site that allows these molecules to engage the huge number of distinct antigens that may be encountered. Variable regions are generated through somatic gene rearrangement, known as primary diversification. Antibodies serve a wide array of immune functions, including binding to and neutralizing toxins and pathogens and making them susceptible to receptor-mediated endocytosis by phagocytic cells (opsonisation).

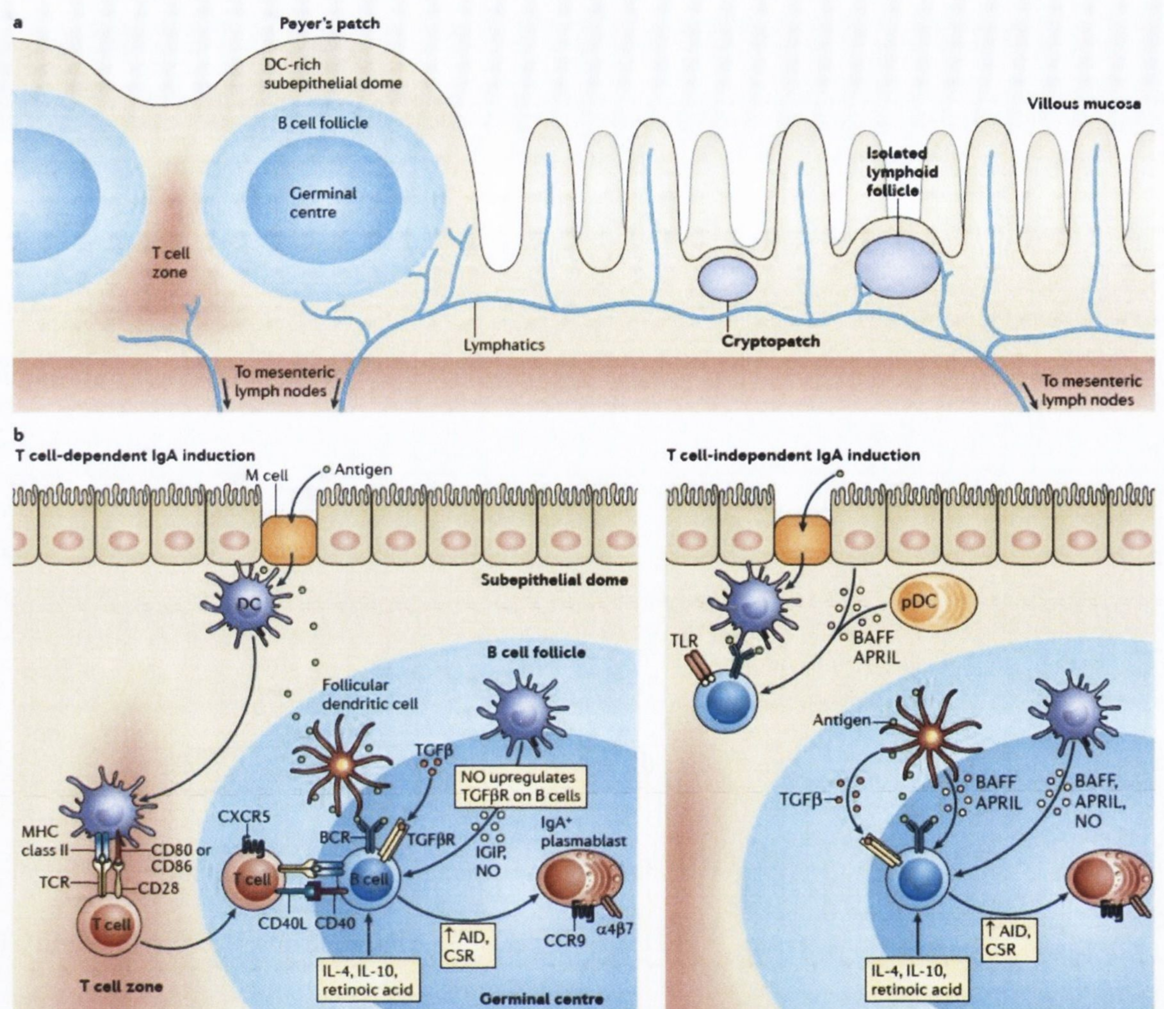


Figure 1.11 – The organization of IgA Induction. a) The anatomical sites of IgA induction are found in the Peyer's patches (PPs), the cyroptopatch-isolated lymphoid follicles (ILFs) and mesenteric lymph nodes (MLNs). b) IgA induction can be achieved via a T cell-dependent (left) and independent (right) mechanism. The T cell-dependent mechanism begins when antigens are transcytosed by microfold (M) cells and encountered by dendritic cells (DCs) in the subepithelial dome (SED) of the PPs. DCs then enter the interfollicular T cell zones where they activate naïve T cells initiating their differentiation into effector T cells. These effector T cells then enter B cell follicles and secrete IgA-inducing cytokines. Further B cell stimulation is achieved by direct contact with T cells via CD40 ligand (CD40L) interaction and cytokines, which enhance expression of activation-induced cystidine-deaminase (AID) which promotes class-switch recombination (CSR) in B cells towards the IgA phenotype. Furthermore, nitric oxide (NO) released by DCs up-regulates the expression of transforming growth factor-beta receptor (TGF-βR), which potentiates CSR towards IgA secretion. During T cell-independent IgA induction, AID expression is achieved through innate mechanisms including toll-like receptor (TLR) signalling and the CD40L- associated cytokines the B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) produced by activated DCs in the absence of T cell stimulation. (Taken from Pabst, O. 2012).

Antibodies also facilitate complement activation and pathogen identification. IgM is a pentameric antibody, and one of the first classes to be expressed on the surface of and secreted by activated B cells, after which its blood serum titres decrease. Its purpose is to neutralize toxins, the agglutination of antigens and is a very effective complement activator. IgA, which is present in mucosal secretions and breast milk is a very important antibody involved in mucosal immunity. It is a dimeric antibody, composed of two IgA monomers linked by a J chain. IgA acts at mucosal membranes to agglutinate and neutralize antigens, trapping them in the mucus, which can then be expelled. It also confers passive immunity to breast feeding infants. IgD is a monomer primarily present on the surface of mature naïve B cells, but its function remains to be fully determined. IgE is a monomer which triggers the release of histamine from mast cells, eosinophils and basophils and plays an important role in parasite infections and allergy. IgG is the most abundant antibody class present in the serum and tissue fluids. It promotes opsonisation, neutralization and agglutination of antigens and activates complement. It can also cross the placenta, thus inducing passive immunity in the foetus. IgG is further subdivided into IgG1, IgG2, IgG3 and IgG4 the latter of which is only found in humans.

1.7.1 – Secretory IgA

The population of lymphocytes found in the LP contains large number of CD4+ T cells and plasma cells. This important population of IgA producing B cells pump out large quantities of the frontline defensive molecule of type I mucosae, SIgA. SIgA is the main Ig class produced in type I mucosal tissues, especially in the GIT which produces amounts in the kilogram range annually [82]. Produced in the LP of the intestine as a dimer, the IgA binds to the polymeric Ig receptor (pIgR) located on the basal side of intestinal epithelium which then, via an active mechanism, transports the IgA molecule from the LP, through the epithelial cell across into the intestinal lumen [83]. SIgA forms a critical part of the intestinal immune system both in

protection from harmful pathogens and in homeostasis. It is especially suited to its role in the intestinal immune system as SIgA is highly protease resistant, and thus can perform its function in the presence of the many digestive enzymes located in the gut. SIgA exerts its functions by neutralizing bacteria toxins, interfering with adhesion molecules expressed by pathogens and delivering harmless and invasive pathogens antigens out of the LP back into the lumen of the gut were they are excreted together with the digestive wastes. SIgA secreted into the lumen of the intestine functions by limiting direct contact of both commensal and pathogenic bacteria and viruses with the gut epithelium, and together with other anti-microbial factors generates a "no go zone" [27]. SIgA indeed has been shown to limit the penetration of colonizing bacterial in the intestine [47, 84].

Naïve B cells in the MALT are precursors for IgA+ B cells after an event known as class switching. Class switching from IgM to IgA is induced by antigen stimulation in the presence of TGF-β, IL-10 and BAFF. This can occur in both the presence and absence of T cells deemed T cell dependent or independent B cell activation, both of which lead to IgA production (Figure 1.11). Regardless of the method of IgA induction, there exists a definitive bias towards both the differentiation and promotion of survival of IgA+ plasma cells in the intestinal LP. This may be explained by various resident cells, which contribute to a milieu of cytokine and effector signals that together generate a microenvironment conducive to IgA+ plasma cell survival (Figure 1.12).

It is thought that T cell dependent IgA production plays an important role in pathogen and vaccine mediated immune responses while T cell independent class switching is an important component of mucosal tolerance and in the maintenance of homeostasis with the microbiota. SIgA has been show to function largely by neutralizing toxins and other pathogen produced effector molecules. SIgA also neutralizes moieties which are involved in pathogen adherence to intestinal cells thus preventing their attachment, in the intestinal lumen.

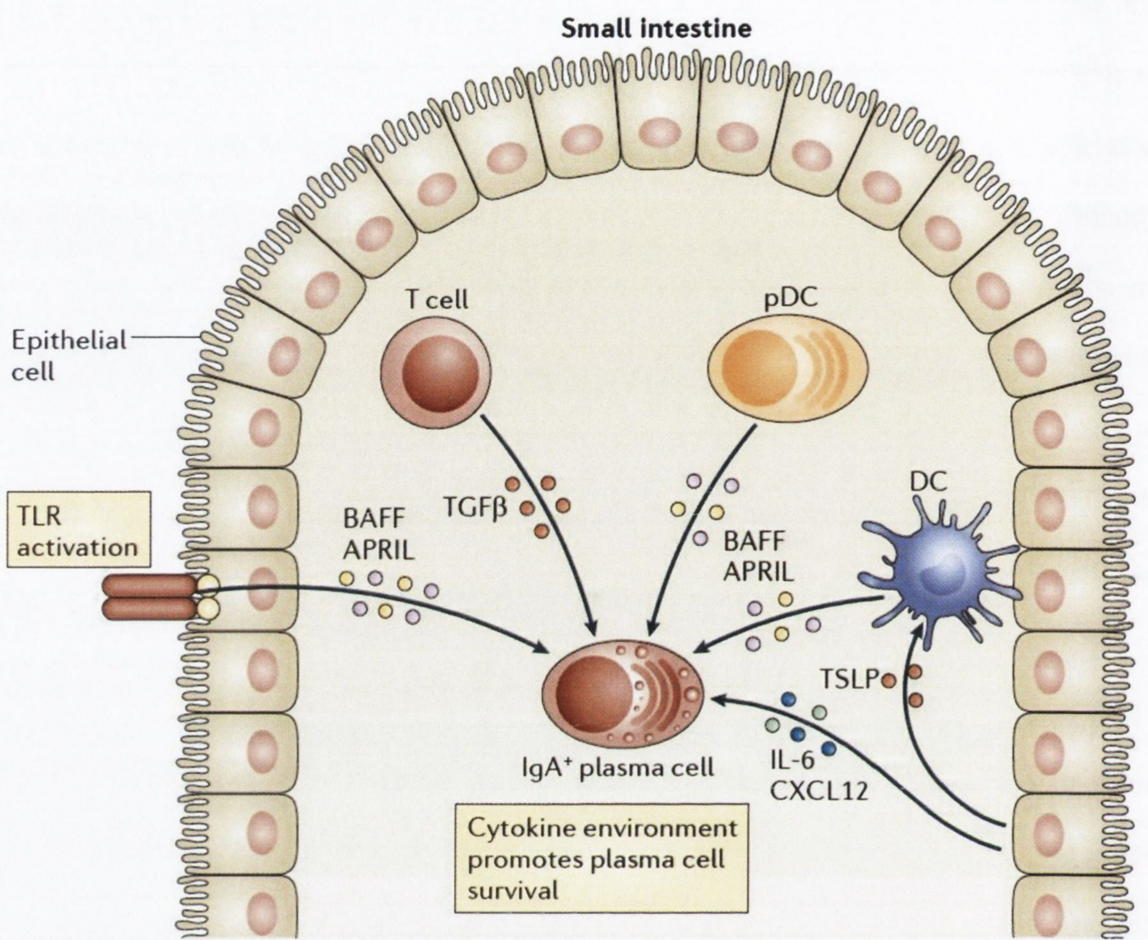


Figure 1.12 – Specific signalling pathways and secreted cytokines contribute to a microenvironment within the lamina propria which is conducive to plasma cell survival. Dendritic cells (DCs) and intestinal epithelial cells (IECs) secrete B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL). IECs also secrete interleukin-6 (IL-6), CXC-chemokine ligand 12 (CXCL12) and thymic stromal lymphopoietin (TSLP) which up-regulates APRIL expression in DCs, further augmenting its secretion. T cells also contribute transforming growth factor-beta (TGF-β) into this cytokine micromillieu thus creating a survival nice for IgA+ plasma cells within the lamina propria. (Taken from Pabst, O. 2012).

Furthermore, SIgA may protect against intracellular pathogens and pathogens and toxins in the LP by binding to them and excreting them back across into the lumen of the intestine. Bound bacteria and toxins can then be excreted by the peristaltic movements of the gut by becoming trapped in the mucus (Figure 1.13). The protective role of natural SIgA in the gut

may in part be due to its cross-reactivity with bacterial antigens, especially polyclonal ones such as LPS. Exclusion and excretion of antigens may not be the only function of SIgA, but rather it may enhance local immune responses by delivering antigens to M cells and later to DCs for presentation [85]. Protection against mucosal infection is often mediated by SIgA, thus a vaccine against such pathogens should be capable of driving high levels of antigen-specific SIgA secretion at the target mucosa.

1.8 – Barriers to Mucosal Vaccination

Many current vaccination methods do not induce strong mucosal responses. In order to elicit a mucosal response, a vaccine should be directed to mucosal tissues. Direct mucosal vaccination however has presented many challenges.

1.8.1 – Mucus Membranes

Mucosal tissues are covered in a thick layer of mucus (Figure 1.13). Mucus is a highly viscous and heterogeneous microenvironment that forms an additional layer of defence against pathogens and has a viscosity that is 100 to 10,000 times greater than that of water [86]. However, this also proves to be a layer of difficulty when mucosal vaccines are concerned. An effective mucosal vaccine must deliver its antigen and adjuvant intact through the harsh environment overlying the mucosal barrier. Vaccines administered at a mucosal site can become diluted by the movement of mucus resulting in a less than effective dose being delivered to a particular site at the epithelium, a challenge which particularly pertains to oral vaccination [12]. Furthermore, a vaccine may even become trapped within the mucus layer and be subject to excretion or damage by proteases [12]. Interestingly, work conducted on human cervicovaginal mucus has revealed that mucus contains pores that are often significantly larger than human viruses such as human immunodeficiency virus (HIV) [87] but the mucus still inhibits their diffusion (Figure 1.13 a).

It is proposed that hydrophobic and electrostatic interactions between the outer- membranes of such viruses and the mucin fibres in the mucus induce alternations of the mucus-microstructures thus trapping viruses [88]. With these findings in mind it may be useful to design carriers for mucosal vaccines which either take advantage of these pores or the electrostatic properties in the mucus. However, both muco-adhesion and muco- penetration may prove useful when designing a vaccine. A vaccine administered to a site of rapid mucus clearance may benefit from quick muco-penetration, whereas a site with lower rates of mucus clearance muco-adhesion may provide additional benefits. To this end it is thought that more hydrophilic carriers with net-neutral surfaces have enhanced muco- penetration, while muco-adhesion can be achieved by highly hydrophobic or positively charged surfaces which interact with the largely negatively charged mucus, thus prolonging the presence of the carrier in the mucus layer [89]. Other possible benefits of muco-adhesion of vaccines besides prolonging the exposure of the vaccine to MALT tissues may also be the limiting of the dilution of vaccine material in the mucus. Work on naturally occurring biomaterials such chitosan (a compound formed by the deacetylation of the chitin) has revealed that its strong muco-adhesive properties stem from the presence of numerous hydrogen-bond forming groups on the compound [90]. Other research has shown that coating particles with a surfactant increases their ability to diffuse through the mucus and enhances their uptake across the mucosal membrane [91]. Depending on the nature of the mucosal tissue where the vaccine will be administered, is important to consider the nature of the mucus overlying the epithelial layer.

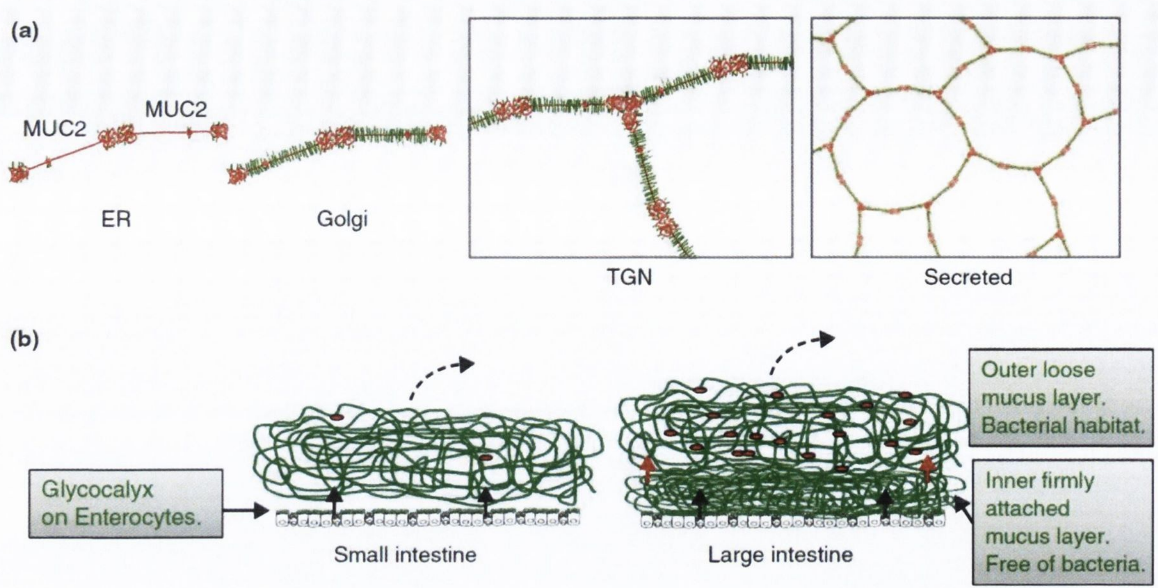


Figure 1.13 – The formation of the MUC2 mucin in the small and large intestine. a) The protein core of the MUC2 mucin is formed in the endoplasmic reticulum (ER) as a dimer. In the golgi apparatus O-glycosylation occurs followed by trimer formation in the trans golgi network (TGN). The secreted MUC2 mucin polymer forms a porous mesh-like structure. b) Goblet cells (Black Arrows) secrete MUC2 mucin which after polymerisation forms the mucus layer. In contrast to the small intestine, the large intestine forms a two-layered mucus. This process occurs as the inner layer of mucus is converted to the outer layer (Red Arrows), which traps bacteria (Red Dots). (Taken from Hansson, G.C. 2012)

1.8.2 – Targeting Antigens

Antigens are internalized, processed and presented at discrete sites in mucosal tissue (Figure 1.7). Even if a vaccine formulation can penetrate the mucus layer overlying this tissue there are no guarantee that it will come into contact with immune sensing areas along the mucosa. Therefore it may be beneficial to target these vaccines or delivery systems in such a way that they are directed towards cells that can initiate the desired immune response. Because of the key role they play in eliciting mucosal immune responses and their position at the frontline of the mucosa, many strategies to target mucosal DCs have been developed. This work which was pioneered by the late Ralph Steinman, who was the first to report these DC specific receptors, would later form the basis for DC-specific targeting strategies [92, 93]. Examples of such DC-specific receptors include CD205 (often called DEC205) and DC-specific

intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) [94]. Expression patterns of different DC-specific receptors varies between different mucosal DC subsets, types of mucosal tissue and mature and immature DCs [30]. One must also consider the intracellular routing of the targeted receptor as these may influence the antigen presentation pathway that follows. Interestingly, it is also possible to specifically target DCs with Ulex europaeus agglutinin I (UEA-1) (Davitt et al, unpublished) which is also used to target M-cells. The dual specificity of the molecule may account for its ability to enhance both the uptake of mucosal vaccines and their immunogenicity [95].

The pivotal role played by M cells in the generation of mucosal immune responses has made them a target for mucosal vaccine delivery. The unique glycosylation pattern displayed by these cells suggested these cells may be specifically targeted with lectins [96]. When UEA- 1, an α-L-fucose specific lectin, was used to target particulate oral vaccines to murine gut M-cells it specifically increased their uptake [95, 97]. Furthermore, a UEA-1 mimetic has also been shown to enhance particle uptake after oral vaccination when targeted to M-cells [98]. This also translated to an enhancement of mucosal vaccine efficacy [99].

1.9 – Mucosal vaccine formulations and delivery systems

An effective vaccine formulation can only elicit a protective immune response when delivered intact and to the right anatomical location. The vaccine must also be formulated in such a way to maximize its immunogenicity. Selecting the right formulation method and delivery vehicle can promote mucosal antigen uptake and immune activation. Often formulating a vaccine in a soluble solution will not be sufficient to elicit a protective immune response as access to sites of antigen uptake might be impeded or diluted my mucosal secretions or in the case of oral food and digestive fluids. Furthermore, shielding an oral vaccine from the many destructive obstacles encountered on its journey from the point of ingestion via the mouth to its arrival in

the intestine. The acids in the stomach and enzymes in the intestines can often degrade a vaccine's components before they have a chance to encounter immune cells and stimulate the desired response, therefore it is wise to carefully select a suitable delivery system to help overcome these challenges.

1.9.1 – Synthetic polymer-based delivery systems

Polymer-based micro and nano delivery systems have seen very steady growth over the past few years and are emerging as a novel platform for the development of innovative mucosal vaccination strategies. Leveraging these technologies enables the circumvention of many of the barriers imposed on mucosal vaccination allowing antigens and adjuvants to be delivered to the desired mucosa. Carriers can be made from a large variety of synthetic and naturally occurring materials such as lipids, polymers, proteins, carbohydrates and inorganic materials. Furthermore, it is possible to manufacture particles of defined size, architecture and with distinct chemical properties in order to target specific cell types, and specific mucosal sites and to suit specific payloads. These delivery systems are broadly classed into two categories, particles and capsules. Particle delivery systems are composed of a solid material to which a vaccine payload is either attached or adsorbed or dispersed within the polymer matrix (Figure 1.14). In contrast, capsules contain one or several reservoirs of a vaccine formulation usually suspended or dissolved in oil- based or aqueous solution encapsulated in a solid or semi-solid shell (an example of a capsule-based delivery system is shown in figure 3.1.2). Often these systems are designed to deliver low molecular weight drugs or biological molecules to effector or disease sites that are released upon degradation, swelling and diffusion from the polymer or change in electrostatic interactions. These properties make capsule and particle-based delivery systems especially suitable for mucosal vaccination. An example of a capsule-based oral drug delivery systems include the Single-Multiple Pill® (SmPill), a novel system for the local intestinal delivery of anti-inflammatory drugs developed by Sigmoid Pharma.

The details of this system will be introduce in depth in chapter 3, including the adaption of the SmPill as a delivery vehicle for oral vaccines.

Particulate mucosal delivery systems have an incredible versatility, due in part to the large number of compounds and polymers and methods for synthesis available. This diversity of materials has led to the development of several classes of particulate delivery systems such as solid matrix nano and microparticles composed of natural or synthetic polymers or inorganic compounds, micelles and dendrimers (Figure 1.5). Polymers can broadly be divided into biodegradable and non-biodegradable of which some are naturally occurring (chitosan, alginate, glucan and albumin), synthetic (polyesters, polystyrene and poly(amino acids)), polymer blends and copolymers [100]. Many of these particulate carrier systems have already seen clinical application for drug delivery and their role in improving the efficacy of mucosal vaccination is the topic of much investigation [100]. Some of the merits of particulate systems which make them applicable for mucosal vaccine delivery include their ability to protect the payload from degradation, the ability to penetrate mucosal barriers, controlled and targeted release of the payload so that both antigens and adjuvants achieve maximum efficacy. These are all controllable by altering particle size, surface chemistry and 3D architecture and the ability to target, control release of antigens and adjuvants to specific cells. The size of a particle plays a critical role in determining the dose of both antigen and adjuvant that can be loaded onto each respective particle. Particle sizes can range from millimetres to nanometres. Early data may suggest a role for particle size in determining the type of T cell response elicited after administration (Hearnden, C.A *et al*, unpublished).

Micelles consist of amphiphilic block copolymers displaying both hydrophobic and hydrophilic segments which allows for differences in solubility (Figure 1.15 d). These unique properties drive the self-assembly of these nanoparticles into core-shell architectures which then allows for a payload to be delivered inside the core or attached on the outside to the shell

depending on the electrochemical properties of the vaccine formulation [101]. Unfortunately, micelles can often dissociate when diluted, leading to loss of the payload, however this feature can be exploited using new polymers which allow for the intentional release of a payload in a controlled-release fashion. Micelles can also be synthesized in the nanometer range, allowing for the engineering of delivery vehicles that can potentially penetrate mucus with ease and be taken up by mucosal effector cells, making them an attractive vehicle for mucosal vaccine delivery. Dendrimers, similarly to micelles can be either used as non-covalently loaded encapsulated vaccines or covalently- liked vaccine-dendrimer conjugates [102]. Compared to micelles, dendrimers offer greater stability due in part to the formation of covalent bonds, forming the branched polymer network (Figure 1.15 a).

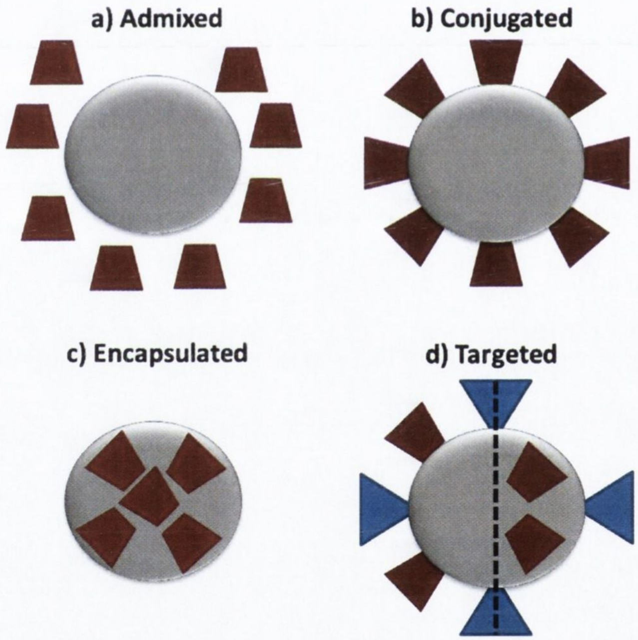


Figure 1.14 – Synthetic and naturally occurring particles have multiple applications as mucosal vaccine delivery vehicles and adjuvants. a) Admixing involves the administration of antigens and adjuvants (Red) together with the particles (Grey) as a mixture. Particulate adjuvants such as alum are administered in this manner. b) Chemically attaching antigens and adjuvants to particles is known as conjugating. c) Incorporating antigens and adjuvants within a particle structure in order to shield them during delivery is called encapsulation. d) Targeting particles involves the attachment of a molecule that confers onto the particle specificity for a particular cell or tissue with the ultimate goal of enhancing uptake and cell specificity and improving the immunogenicity and adjuvanticity of antigens and adjuvants. Both conjugated and encapsulated antigens and adjuvants can be delivered by this method.

Particles are often fabricated using emulsion techniques. This technique involves the generation of an emulsion of the polymer and a solvent which upon solvent removal leads to particle formation [103]. Another fabrication method is gelation of polymers suspended in emulsion droplets when the suspension is exposed to a change in temperature, pH or addition of cross-linking agents [104]. The most widely produced nanoparticles are manufactured from the copolymer poly(lactic-co-glycolic acid) (PLGA), which is the most widely used system in both drug and vaccine delivery. An impressive controlled release and safety profile has led to PLGA being approved by the Food and Drugs Administration (FDA) in the USA [105]. PLGA nanoparticles have been shown to penetrate and distribute through mucosal tissue after local administration, most likely via cellular trafficking [106]. Another feature of PLGA nanoparticles that makes them an attractive delivery system for mucosal vaccines is that the surface chemistry of these particles is easily modifiable and can be altered to increase diffusion through mucus and uptake by M cells [91, 105, 107]. The architecture of PLGA nanoparticles also allows for the encapsulation of antigens and adjuvants, thus providing a protective function (Figure 1.14) [107].

Biodegradable systems synthesized from natural or synthetic polymers can be constructed in sizes ranging from about 100 nanometres upwards. Uptake of these particles by mucosal tissue is reported to be highly dependent on size and on the tissue involved. As mentioned previously the ability of particles to navigate through the pores in the mucus is dependent on both size and surface electrochemical properties [87, 89]. Studies have shown that particles ranging in size from 300nm up to 1000nm are taken up by PPs [108]. Interestingly, particles taken up at mucosal surfaces can also be transported via the lymphatic system to draining lymph nodes; however this is also dependent on particle size [109, 110].

Both synthetic and naturally occurring polymeric particulate delivery systems can be constructed in such a way that they have a specific surface chemistry and composition. This

allows from them to be specifically engineered to overcome mucosal delivery barriers, interact with specific cell types and exert desired immunomodulatory functions. Altering the hydrophobicity or hydrophobicity of a particle can not only determine its interactions with mucus, but hydrophobic particles have been shown to engage DAMP receptors leading to activation of innate cells via this intrinsic adjuvant property [111]. Complement mediated immune activation by particles can also be achieved by adding hydroxyl groups to the surface which interact with the complement activating protein C3b [110]. PAMPs can also be attached to the surface of particles to enhance innate activation via TLRs [105].

The principle of targeting vaccines was introduced in section 1.8.2. By selecting the right polymer it is also possible to determine the route of antigen presentation, either MHC class I or MHC class II. Utilizing nanoparticles made from propylacrylic acid and polycations Flanary et al showed that it is possible to promote the escape of antigens from endosomes into the cytosol for MHC class I presentation which enhanced T cell activation [112]. Such systems rely on polymers that are sensitive to pH or redox potential which can alter the intracellular fate of an antigen. Acrylic acid is an example of a pH sensitive compound, which inside the cell acts to destabilize the membrane of the endosome in a pH dependent manner, [113]. Polycations on the other hand destabilize membranes by osmosis as during acidification the polymer sequesters the protons in the endosome, which leads to an osmotic imbalance, swelling and rupture and release of the endosome contents into the cytosol [114]. Redox-sensitive polymers are also able to distinguish between the redox potentials in different cellular compartments such as endosomes and lysosomes which are reductive and oxidative respectively [115].

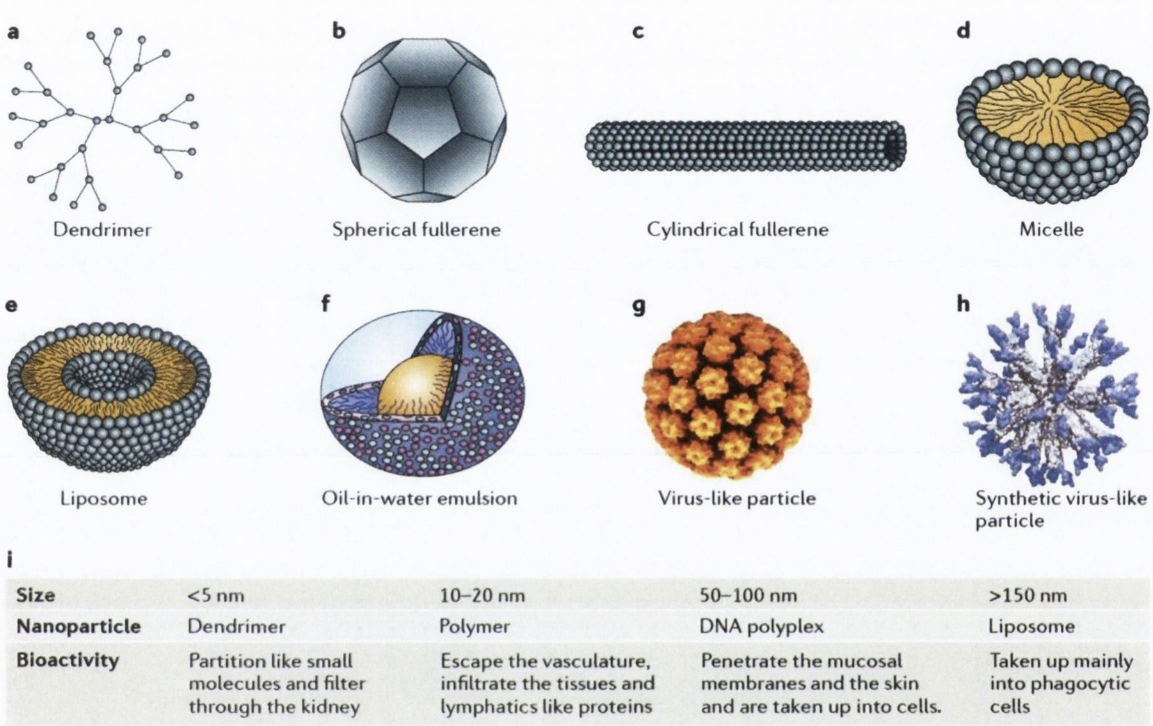
1.9.2 – VLPs and Virosomes

An interesting delivery method for viral subunit antigens are virus-like particles (VLPs) and virosomes. VLPs and virosomes are composed of immunogenic viral components which self-assemble into 3D structures that maintain not only the unique peptide sequence of the antigen, but also the antigenic structure. VLPs contain one or more viral capsid proteins which make up the protective outer shell of a virion, and are usually expressed in a eukaryotic cell (Figure 1.15). An example of a clinically and commercially viable VLP vaccine is the hepatitis B (HepB) vaccine. The vaccine is a product of the self-assembly of HepB surface antigen expressed in recombinant yeast cells.

Another licensed VLP vaccine is the human papillomavirus (HPV) vaccine which was also the first prophylactic vaccine against a carcinogen. Produced by Merk & Co, Gardasil® is a self-assembling VLP providing quadrivalent protection against genitournial HPV infection. It is composed of the L1 capsid protein of HPV-6, -11, -16 and -18 types expressed by recombinant yeast cells [116]. Interestingly this vaccine, which is administered in adolescents via parenteral injection, elicits strong protection at genital mucosal surfaces via serum IgG production which transudes across the epithelium. This IgG binds and neutralizes HPV virions and thus protects against infection [117-119]. Despite forming particulate structures, VLPs require the addition of adjuvants to ensure a robust immune response is elicited.

Virosomes differ from VLPs as they are composed of both viral proteins and lipids either viral or otherwise and are quiet similar to liposomes [120]. By far the most effective and advanced virosomes are those constructed from lipids derived from viral, natural or synthetic sources and proteins of the influenza virus. These are referred to as immunopotentiating reconstituted influenza virosomes (IRIVs) and are distinguishable from other liposomes by the inclusion of the influenza membrane proteins hemagglutinin and neuraminidase in the

construct. Despite the obvious application of IRIVs as virosomes for influenza vaccines [121, 122], these have also been shown to be efficacious delivery vehicles for vaccines against hepatitis A [123] and HIV [124].



Size	<5 nm	10–20 nm	50–100 nm	>150 nm
Nanoparticle	Dendrimer	Polymer	DNA polyplex	Liposome
Bioactivity	Partition like small molecules and filter through the kidney	Escape the vasculature, infiltrate the tissues and lymphatics like proteins	Penetrate the mucosal membranes and the skin and are taken up into cells.	Taken up mainly into phagocytic cells

Figure 1.15 – The application of nanotechnologies as delivery vehicles and immune stimulating agents. There are many different types of nanoparticles; however, they can be classified into three broad categories; nanoparticles (a-c), nanoemulsions (d-f) and virus-like particles (g-h). (a) Dendrimers are branched nanoparticles. The carbon based spherical (b) and cylindrical (c) fullerene. These structures are especially suited to mucosal delivery as they can often penetrate though tissues and mucus with ease (i). Nanoemulsions constitute immiscible components such as oil-in-water emulsions (f) or lipids that form structures when added to water such as micelles (d) and the double layered liposome (e). Nanoemulsions often possess an intrinsic adjuvant effect as well as aiding in the solubility of hydrophobic molecules in a hydrophilic environment. Virus-like particles are composed of self-assembling structures that are either viral capsid proteins (g) or synthetic variants that retain self- assembling characteristics (h). A description of the relationship between nano particle size and bioactivity is found in (i). (Taken from Smith, D.M. et al, 2013)

Although the landscape for VLP and virosomes vaccines is currently sparse, both technologies represent an exciting platform to support the development of novel mucosal vaccines. The advantages of VLPs and virosomes for mucosal delivery are their small size and the variable composition of their surface chemistry, which allows for the selection of the correct electrochemical properties to ensure desirable interactions with membranes and mucus. VLPs and virosomes can also be engineered to incorporate proteins with a variety of beneficial functions from targeting, enhanced antigenicity and immunogenicity to co-stimulatory molecules by inserting the genes encoding these peptides into the recombinant vector's genome. However, challenges for both systems also exist. Both VLPs and virosomes suffer from difficulties in scaling up production and both require extensive purification and quality control. Both systems are also difficult to formulate and batches suffer from poor reproducibility compared to synthetic polymer-based systems. In addition, VLPs require the addition of adjuvants in order to elicit an immune response. However, virosomes do not require the addition of adjuvants and so may be the more attractive of the two options as mucosal delivery vehicles [125].

1.10 – Mechanisms of adjuvanticity

Since their first incorporation into vaccines, the immune potentiating mechanisms behind injectable adjuvants have been intensively studied (Figure 1.16). For instance, while the precise mechanism of adjuvanticity behind alum remains to be elucidated, a role for NLRP3-has been postulated [126]. Furthermore, other particulate adjuvants seem to be dependent of the activity of the NLRP-3 inflammasome [127]. A well-established read out of NLRP-3 activation is IL-1β production from DCs following TLR stimulation [128]. The mechanisms behind TLR-agonist based adjuvants are also well categorized where the engagement of these receptors leads to the activation of pathways to potentiate the immune response via the

cytokines produced [129, 130]. However, for unknown reasons many highly efficacious injectable adjuvants do not function well or even at all when administered orally.

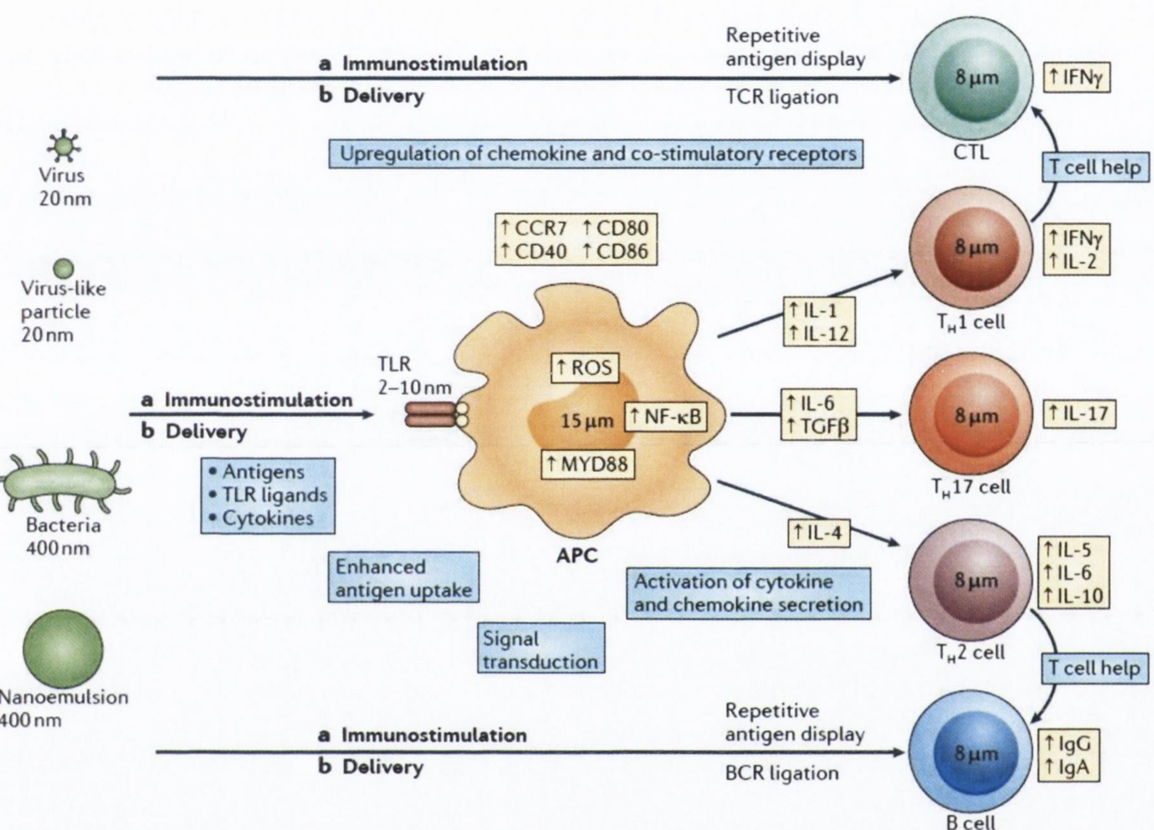


Figure 1.16 – Activation of the immune system by nanoparticles. Nanoparticles may directly activate the immune system (a) or deliver compounds which then result in the immune responses (b) or both. Nanoparticles can influence the expression of receptors and the secretion of cytokines and so can be used to modulate an immune response. (Taken from Smith, D.M. et al, 2013)

1.11 – Mucosal Adjuvants

Selecting an appropriate adjuvant for a vaccine formulation is as critical to achieving a successful result as choosing the antigen itself. The choice of adjuvant will determine both the short and long term consequences of a vaccination but also the quality of the immune response elicited [129, 131]. Although OPV does not require an adjuvant to elicit mucosal protection, WCK and subunit vaccines often do not induce a sufficiently strong immune response alone and thus require the addition of an adjuvant to enhance immunogenicity. Furthermore, the incorporation of adjuvants in vaccine formulations may augment their ability to elicit antibodies with higher avidities and greater neutralization potential than antigen alone [132]. Mechanistically many injectable and mucosal adjuvants exert their effects by similar pathways. Unfortunately some adjuvants that do display mucosal adjuvanticity are often too reactogenic for use in humans. Currently only two adjuvants have been approved in the US for clinical use, aluminium salts (alum) and alum with the TLR4 agonist monophosphoryl lipid A (MPL), while in Europe alum and the oil-in-water emulsion MF59 are licensed for use in vaccines. Alum has been in use for over 80 years, when in 1927 Alexander Glenny discovered that the addition of aluminium containing salts (Al[OH]3 and AlPO4) or Alum for short could enhance the protective efficacy of diphtheria toxoid. Alum has since been administered to millions of patients even though the mechanism of action is still subject for debate and controversy [133]. Recently the oil-in-water emulsion adjuvant AS03 was licensed for the H1N1 flu vaccine during the 2009 pandemic.

A diverse range of adjuvants have been and are currently being tested for their ability to induce strong mucosal immune responses. Mucosal adjuvants based on modified bacterial toxins such as heat-labile enterotoxin (LT) from Enterotoxogenic *Escherichia Coli* (ETEC) and cholera toxin (CT) from *V. cholerae* have shown great promise as mucosal adjuvants while immunostimmulatory adjuvants either TLR agonists such as MPL, CpG and other

adjuvants such as QS-21 and alpha-galactosylceramide (α- Galcer) have also been evaluated. Particulate adjuvants including emulsions, PLGA nanoparticles (which can serve as both delivery vehicles (section 1.9.1) and adjuvants) and immunostimulating complexes (ISCOMs) may also have potential in mucosal vaccines although currently evidence in their favour is limited particularly for the oral route.

1.11.1 – Mucosal adjuvants based on toxins

By far the most potent category of mucosal adjuvants discovered to date are those based on bacterial enterotoxins, CT and LT. Both toxins share a significant sequence homology [134] and are AB5 complexes composed of an ADP-ribosylating A1 subunit connected to a donut shaped pentamer of B subunits joined by an A2 linked chain (Figure 1.22) [135]. Direct interaction with DCs is required for the adjuvant activity of CT, which was shown to be dependent on the ability of the B subunit of CT (CTB) to bind to monosialotetrahexosylganglioside 1 (GM1) [136]. The activation of DCs is also crucial for the initiation of mucosal immune responses [137]. Following binding to GM1 the A1 subunit of the holotoxin mediates the ADP-ribosylation of a membrane-bound heterotrimeric guanine nucleotide- binding protein α-subunit (Gsα) which increases the cytoplasmic level of cyclic adenosine monophosphate (cAMP) [138]. In addition to increasing cAMP levels in the cytoplasm of DCs, CT and LT holotoxin or the B subunits alone increased the numbers of DCs migrating into the follicle-associated epithelium (FAE) which increased their ability to capture antigens [139]. Interestingly, CTB was shown to promote IgA production in B cells by DCs via a RA and TGF-β dependent mechanism [140], which may account for its use as a tolerance inducing adjuvant [141]. Due to the high level of structural and sequence homology between CT and LT it is plausible to speculate that both toxins exert their adjuvant effects by identical or highly similar mechanisms. CTB is also capable of delivering LPS in a manner which activates caspase-11 leading to the production of pro-inflammatory cytokines by innate

cells in a mouse model [142], but the significance of this finding has yet to be correlated to the adjuvant activity of CT. Unfortunately, despite the success of pre-clinical studies these are too toxic in their native from for use in humans. GM1 is ubiquitously expressed on all cells which can lead to unwanted side effects when toxins are taken up by cells other than immune cells. When a nasal VLP-based influenza vaccine adjuvanted with LT was tested in humans, several individuals developed Bell's Palsy (facial paralysis) as a result of LT interacting with GM1 on nerve cells [143-145].

This led to the generation of toxin mutants with attenuated enzymatic activity by site directed mutations in the A1 subunit [146]. Site-directed mutagenesis of LT led to the generation of potent, yet safer (than wildtype) mutant toxin adjuvants ((Figure 1.17 a). LTK63 and LTR72, both developed by Novartis, each contain a one amino acid mutation that abolishes or significantly reduces their ADP-ribosylating activity respectively while maintaining their ability to elicit immune responses (Figure 1.17 a) [147]. The LT mutants LTK63 and LTR72 were extensively tested as intranasal mucosal adjuvants. In several studies whole cell mucosal vaccines adjuvanted with LT mutants led to protection against challenge with the mucosal pathogens *Bordetella pertussis* [148] and *Streptococcis pneumoniae* [149]. Oral and intranasal administration of a HIV-1 p55 gag subunit vaccine adjuvanted with LT mutants elicited strong cytotoxic T cell responses [150]. When mice were orally vaccinated with recombinant CTB and LTR72, strong intestinal IgA titres were elicited and mice were also protected against oral challenge with CT (Davitt et al, unpublished). Although the precise adjuvant mechanism of action of LT and CT mutants remains to be elucidated to it is thought to involve both enhanced permeation of antigens across mucosal epithelial barriers and an increase in antigen presentation and activation of APCs [151, 152]. Enhanced M cell targeting is also observed in vaccines adjuvanted with LT and CT [28]. However, when tested in clinical trials and despite lacking all enzymatic activity [147], LTK63 caused the development

of Bell's Palsy in a number of recipients of an intranasal vaccine. This led to regulatory authorities cautioning against the intranasal use of GM1 binding adjuvants [153]. A double mutant LT (dmLT) was also developed. The double mutant shows great promise for mucosal application as not only does it effectively stimulate mucosal immunity [154], it also exerts no enterotoxicity, even at high doses, and is currently being assessed in clinical trials (Clements, J. *Personal Communication*). Moreover, dmLT is rendered much more resistant to trypsin degradation, and this together with its effective adjuvant activity and improved safety profile make it an ideal candidate for an oral vaccine adjuvant. It is also hoped that the potential success of dmLT may renew confidence in CT and LT mutant adjuvants with regulatory authorities as mucosal adjuvants, especially as the danger of neurotoxicity may not be a problem with oral application.

Another strategy employed when designing mucosal adjuvants is to take the best functions of a bacterial toxin, while discarding the troublesome ones. In order to overcome the issues associated with toxicity Nils Lycke's group developed a non-toxic CT derivative (Figure 1.17 c). This unique adjuvant is a fusion protein composed of the A subunit of CT (CTA) which retains 100% of its enzymatic activity. To this a dimer of the D-fragment of the Staphylococcus aureus protein A is attached to form the adjuvant CTA1-DD [155]. CTA1-DD has already been extensively tested with positive results from variety of different vaccine candidates [156]. By exclusively using the CTA portion of CT, CTA1-DD is also unable to bind to GM1 which has greatly enhanced its safety, and no side effects were observed in tests in animal models and non-human primates post nasal administration [157, 158].

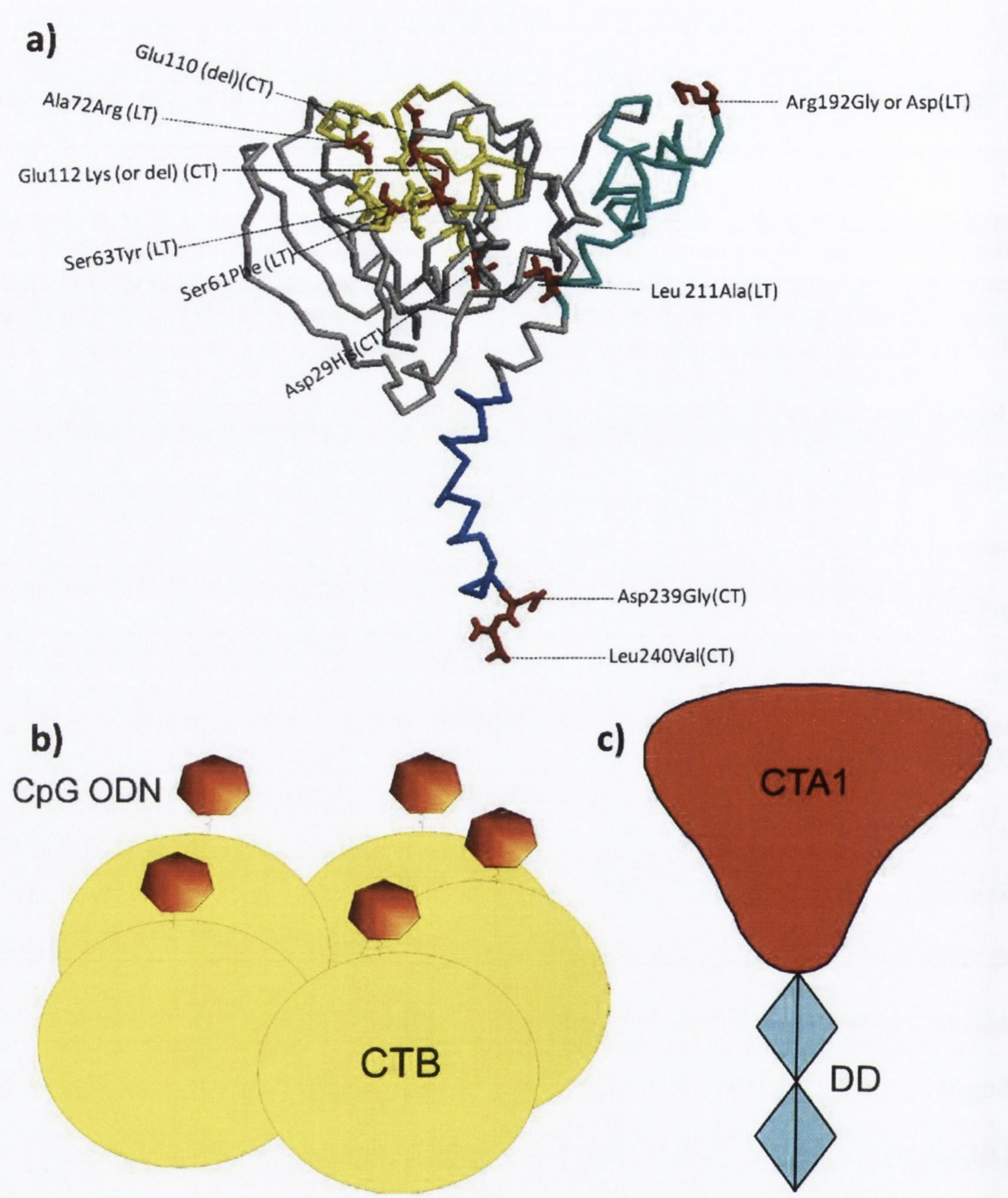


Figure 1.17 – Adjuvants based on modified enterotoxins are potent mucosal adjuvants. a) Amino acid substitutions and deletions in the A subunit of both cholera toxin (CT) and heat-labile toxin (LT) have been shown to reduce toxicity while retaining the molecule's adjuvanticity and an intact B subunit (not shown). b) CTB pentamers coupled to the toll-like receptor (TLR) 9 activating oligonucleotide CpG have been shown to form an adjuvant-active complex. c) CTA1-DD is a unique fusion protein composed of the intact and un-mutated A subunit of CT (CTA1) and a dimer of the immunoglobulin-binding protein DD, a derivative of Staphylococcus aureus protein A). (Taken from Sánchez et al, 2011)

1.11.2 – TLR agonists as mucosal adjuvants

Several attempts have been made to engineer ligands which engage TLRs of the innate immune system in an attempt to develop new adjuvants. TLRs are often formulated into oil-in-water emulsions to increase their efficacy. IC31, a TLR signalling adjuvant is actually a particulate adjuvant composed of a TLR9 activating oligonucleotide (ODN1a) and a cationic peptide (KLK) which aggregate together to form a nanoparticulate emulsion [159]. Several of these TLR agonists have been incorporated into mucosal vaccine formulations. Other TLR9 ligands include CpG, a widely used oligodeoxynucleotide in both vaccine models and clinical trials and has shown mucosal efficacy [160]. MPL and flagellin were also tested as mucosal adjuvants and displayed moderate potential [161]. Many synthetic variants of naturally occurring PAMPs have been developed which are designed to elicit similar cytokine profiles as their wild-type brethren while exhibiting a greatly enhanced safety profile [159, 162]. Lipopolysaccharide (LPS), a structural component of the outer membrane of gram-negative bacteria, is a ligand for TLR4 which is present on most innate immune cells and on B cells. Upon detecting LPS, B cells become activated and promote T cell-independent antibody production. This can enhance polymeric SIgA secretion at the mucosae, which can bind to polymeric antigens such as bacterial polysaccharides, which may play an important role in homeostasis with the microbiota [163, 164]. However mucosal adjuvant screening work conducted by McNeela *et al* determined that TLR 3, 4, 9 agonists are not effective adjuvants for oral subunit vaccines and failed to elicit strong antibody titres (Figure 3.1.7). Furthermore, two molecules TMX201 and TMX202, both synthetic ligands for TLR7, failed to elicit significant responses against CTB following oral administration (Davitt *et al*, unpublished data) or against a candidate oral WCK *Helicobacter pylori* vaccine (Abautret-Daly *et al*, unpublished data). These data suggest that at least in soluble form, TLR ligands are poor oral vaccine adjuvants.

1.11.3 – Particulate Adjuvants

While many particulate systems are designed with delivery in mind (section 1.9.1), several of these have been show to exhibit intrinsic adjuvant activity themselves. Several types of particulates have been shown to activate the NLRP3 inflammasome in DCs including polystyrene and biodegradable poly(lactide-co-glycolide) (PLG) particles. Furthermore, the methods by which these different particulate adjuvants achieve their adjuvanticity is also quite similar [127]. Additionally, chitosan displays a wealth of other beneficial features including its mucoadhesive properties. Chitosan has been show to interact with tight junction proteins leading to opening of these and increased absorption across the epithelial barrier [165]. The immunostimulatory properties of chitosan have been documented in a variety of different studies. When combined with a TLR agonist, chitosan is capable of activating the NLRP-3 inflammasome, similarly to other particulate adjuvants, and this was also shown to be required for adjuvant activity [127, 166]. When delivered to mucosal tissues chitosan was shown to enhance local AIgA production [167] and local innate responses [168]. When combined with CpG, chitosan was show to elicit strong antigen-specific Th1 and Th17 responses [166]. CpG was also coated onto PLG microparticles, and this was found to enhance its immunostimulatory properties [169]. Combining particulate adjuvants with other types of adjuvant (e.g. TLRs) may be a method to achieve superior mucosal immune responses to vaccination.

1.12 – iNKT cells

Most T cells are known for their ability to respond to peptide antigens presented on MHC class I and II molecules via their TCR. T cell receptors are composed of two peptide chains, which allows for a great amount of genetic variability thus accounting for the polymeric responses of T cells. Each chain can be sub-divided into a variable (V) domain and a constant

(C) domain. The V domain contains three complementarity-determining regions (CDRs) in each chain which together form the antigen binding site in the TCR. CDRs 1 and 2 are encoded by the V gene segments, while CDR3 is encoded at the V and J gene segments for the α-chain and V, D and J gene segments for the β-chain. The variety of combinations of the V, J and D gene segments provide the CDR3 loops with great diversity. T cells can express either a αβ or γδ TCR and recognize their specific cognate peptide antigen only when presented by an appropriate MHC molecule. While initially T cells were studied for their ability to respond to peptide antigens, other T cells have been shown to be able to respond to lipids presented by APCs. Lipids can be presented to TCRs by the MHC class 1 like CD1 family [170-172]. MHC class 1 and CD1 molecules share a common evolutionary origin however CD1 molecules are mostly non- polymorphic and have evolved very different antigen-binding groves to enable them to present hydrophobic lipid molecules [173]. The population of T cells restricted by CD1 molecules were initially found to be involved in tumor immunity, pathogen recognition and autoimmunity [174]. A subset of these CD1 restricted lymphocytes are characterized by their specific restriction by the CD1d isotype of the CD1 family and were termed natural killer T (NKT) cells. These cells are characterized by their expression of cell surface markers associated with natural killer (NK) cells and a TCR, characteristic of conventional T cells. However not all NTK cells express all NK cells markers and so can further be subdivided into subsets. NKT cells are one of the most extensively categorized populations of "innate-like" T cells, and have a well-documented immunomodulatory capacity (Figure 1.18).

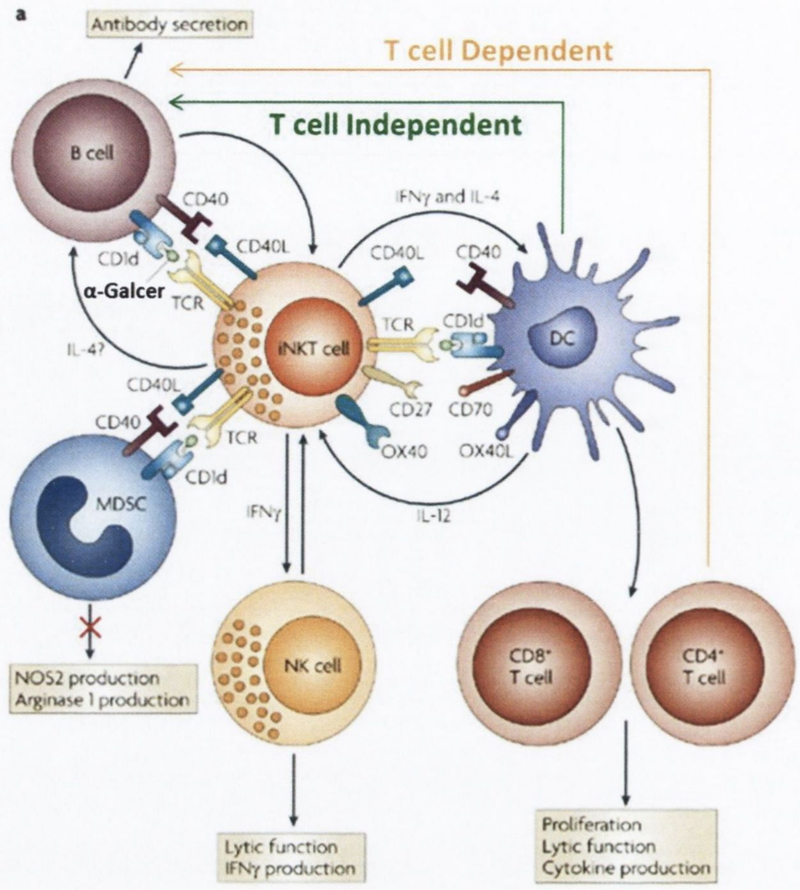


Figure 1.18 – Invariant natural killer T cells are potent controllers of immune responses. Activated invariant natural killer T (iNKT) cells have the capacity to interact with or be interacted with by other cells types and can so send and receive signals that influence the actions of these cells. iNKT cells can activate B cells, DCs and T cells and so induce or suppress immune responses, making them attractive targets as a strategy to modulate an immune response in a favourable direction. (Adapted from Cerundolo, V. et al, 2009)

1.12.1 – CD1d, an unconventional molecule for unconventional antigens

CD1 molecules are a family of antigen-presenting molecules with many structural similarities to the MHC class 1 family [175]. The antigen binding grove of CD1 molecules is defined by two α-helices, which are located above an 8 stranded antiparallel β sheet [176]. Five isotypes of the CD1 molecule family have been identified and are divided into 2 families, group 1 contains CD1a-c and group 2 CD1d [177]. The fifth CD1e molecule has been shown to

enhance the loading of antigens into members of group 1, but as only CD1d bears further significance to this discussion, group 1 molecules will not be discussed in greater detail. CD1d can bind to a variety of different lipid molecules due to variations in the binding groove. However CD1d is essentially monomorphic, in contrast to other MHC classes, it can bind to a large selection of self, non-self and synthetic antigens [178]. The major ligand classes of CD1d antigens include sphingolipids and glycerolipids (Figure 1.19 b). Sphinoglipids were the first class of CD1d antigens to be discovered. Glycosylceramides, a subclass of sphinoglipids have been shown to exhibit strong NKT cell activating potential [179]. Of all the glycosylceramides, α-Galcer is the best characterized. It is composed of a phytosphingosine base (C18) that is N-amide linked to a C26 fatty acid forming the phytoceramide backbone, which carries a α-anomeric galactose sugar (Figure 1.19 a). The structure of the complex between α-Galcer and CD1d has been described (Figure 1.19 c) [180]. α-Galcer is derived from a marine sponge *Agelas mauritianus* and the most commonly available form is known as KRN7000 which comprises an α-linked galactose head group and a ceramide base composed of an 28-carbon phytosphingosine chain and a 26-carbon acyl chain. α-Galcer has been shown to bind to both murine and human CD1d [179].

1.12.2 – Harnessing the immune activating potential of iNKT cells

NKT cells are the best categorized population of CD1d-restricted T cells and express CD161 and the αβ TCR, with varied expression of NK1.1 depending on the subtype [181]. These can further be subdivided into type I and type II NTK cells [181]. These subtypes are characterized by their differential TCR repertoire and antigen specificity [174]. Type I NKT cells express a conserved α TCR chain with an invariant germline-encoded rearrangement that can pair with a limited number of β chains. Because of the invariant α chain, type I NKT cells are often known as invariant natural killer T (iNKT) cells. iNKT cells also respond to α-Galcer. Type II NKT cells on the other hand express a more extensive and variable repertoire

of TCR genes, but do not respond to stimulation with α-Galcer. While both populations are conserved across mice and men, the frequencies of each population varies in both with respect to the subtype and their anatomical location. Type I NKT cells are more abundant in mice [174], however, there is a high level of sequence similarity between the TCR CDR3α and CDR2β loops in humans and mice iNKTs [182]. iNKT cells can also express a variety of other T cell markers such as CD4 but not CD8 [183] although, there is no evidence to suggest that the absence or presence of CD4 contributes to their potential to produce cytokines [184]. iNKT cells are often categorized as innate-like T cells as they also express cell markers associated with innate immunity. They constitutively express CD69 and can rapidly secrete both Th1 and Th2 polarizing cytokines when activated [181]. iNKT cells can produce large amounts of interferon gamma (IFNγ), TNFα, IL-4, IL-10, IL-13, IL-17 and IL-21, and can either promote or suppress cellular response without the need for clonal expansion [181, 185]. iNKT cells also share NK properties such as the ability to produce granzymes and perforins on stimulation. Even though iNKT cells are present in low numbers they play an important role in modulating immune responses to tumors and pathogens, allergy, autoimmune disease, allograft rejection, graft versus host disease and atherosclerosis [174].

NKT cells are resident in the intestines of both mice and humans. These include iNKTs and mucosal NKT (mNKT) also known as mucosal-associated invariant T (MAIT) cells which similarly express an invariant TCR [186]. It has been estimated that 1% of IELs are iNKT cells in both humans and mice [187]. Both human and murine IECs express CD1d [188]. In fact IECs can present both peptide and lipid antigens to conventional and un-conventional T cells in the intraepithelial and LP compartments, and these have also been shown to be capable of activating NKT cells [189]. CD1d is also expressed on intestinal APCs and B cells.

iNKT cells, similar to Th17 cells can produce IL-17 and IL-22 [190]. Furthermore, these IL-17 producing NKT cells were also shown to express the Th17 cell transcription factor RAR-related orphan receptor (ROR) γt and the Th17 cell associated IL-23R [198]. NKT cells have been implicated in the establishment of oral tolerance to antigens in the intestine [190]. However, administration of α-Galcer and subsequent iNKT activation was shown to interfere with the induction of oral tolerance [191]. This finding has led to interest in the application of α- Galcer as a mucosal adjuvant [192, 193]. Increased migration of LP-DCs to MLNs was observed after oral administration of α-Galcer, suggesting that the adjuvant can increase CCR7 expression by LP-DCs [194]. Furthermore, LP-DC activation can be enhanced through cytokine production by activated iNKT cells [195]. During a screen of compounds with anti-tumor activity, α-Galcer was shown to exhibit strong tumor suppression via an NKT-dependent IFNγ-mediated mechanism [189, 196, 197]. Problems with cell anergy after repeated administrations of α-Galcer have been reported however, this is most likely due to the contrasting effects of the release of both Th1 and Th2 cytokines by activated iNKT cells, which have led to disappointing results in clinical trials [198, 199]. When α-Galcer is presented by different APCs, varying cytokine profiles are secreted by the activated iNKT cell which may suggest novel immunomodulatory strategies [200]. The unique modulatory properties of these cells has driven increasing interest in the therapeutic potential of a-Galcer and other novel iNKT cell activators [201].

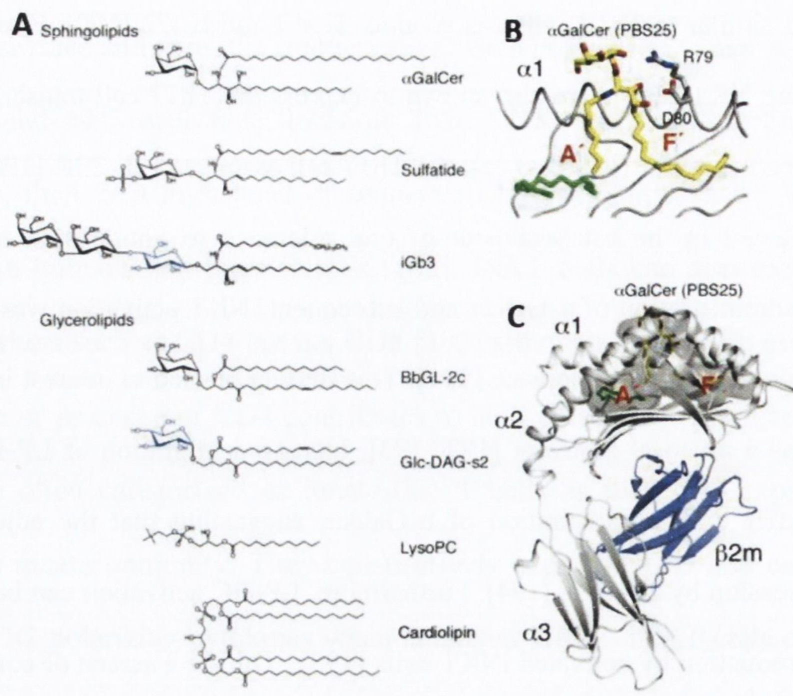


Figure 1.19 – Ligands of CD1d and antigen presentation by CD1d. A) Some of the lipid antigens recognized by CD1d-restricted T cells. B) Shows the antigen-binding groove of CD1d containing α-Galcer (yellow). (C) This cartoon represents the CD1d-β2m heterodimer (CD1d is in grey) with α-Galcer bound (yellow) in the antigen-binding groove. (Taken from Girardi et al, 2012)

1.13 – Infectious Diarrhoea

Diarrhoea caused by pathogens is a significant and major global health problem. In the developed world it accounts for significant morbidity but in the developing world it also causes high levels of mortality. In both developing and developed countries children under 5 are the high risk group [202]. While oral rehydration therapy has reduced the overall fatalities cause by diarrhoeal disease, the long term damage caused during disease episodes is still a cause for concern [202]. During the first two years of life infants undergo a significant period of brain development. If during this critical period of development children suffer from diarrhoeal disease, the impaired absorption of nutrients during these episodes, which often occurs repeatedly, can result in long term developmental disabilities. Stunted growth (often by 8 cm at age 7), lower levels of physical fitness, and impaired cognition which adversely

affects school performance (often a loss of 10IQ points) can have serious implication for children in adolescent and adult life [203]. The two most common diarrhoeal diseases detected globally are caused by ETEC and *V. cholerae*, the causal agent of cholera. These two pathogens, their global impact, disease characteristics and strategies to overcome them will now be discussed in more detail.

1.13.1 – Pathogenic *E.coli*

Identified in 1885, *E.coli* is a bacterial species which constitutes a large proportion of the intestinal commensal population, exists in symbiosis with its host providing essential nutrients such as vitamins and prevents colonization of the intestine by other pathogenic bacteria. However, *E.coli* can acquire an array of mobile genetic elements to provide it with an arsenal of molecular weapons, turning it into a highly adaptable and effective pathogen.

The genomes of pathogenic *E.coli* are influenced by horizontal gene transfer (HGT) which enables rapid dissemination of new traits to recipients. The acquisition of such traits plays a key role in establishing the fitness of an organism to its environment [204]. Pathogenicity islands (PAIs) are clusters of virulence genes often found on plasmids or integrated into the genome in pathogenic bacteria. These PAIs are often flanked by mobile genetic elements, which enable the sequence to be inserted near tRNA genes. Many of the virulence traits associated with *E.coli* are carried on PAIs and plasmids providing able opportunity for HGT, allowing recipient bacteria to occupy new niches and hosts. These genetic properties are thought to be responsible, at least in part for the diversity amongst *E. coli* pathovars [205].

The contribution of these *E.coli* isolates to the global disease burden is staggering, causing significant morbidity and mortality. Pathogenic *E.coli* are the causative agent of a variety of diseases, infecting a variety of tissues from the intestines, urinary tract, bloodstream to the CNS. However, for the purpose of this introduction the focus will only be on the enteric

strains. The range of the causative strains of E.coli that cause disease can be broadly categorized into either diarrhoeagenic or extraintestinal [206]. The diarrhoeagenic class can further be classified by the various virulence genes that are expressed and the mechanism of their pathology. Although several virulence strategies are shared between all strains; adhesion to the host tissue, evasion of host immune recognition and manipulation of physiological processes, each strain has its own characteristic traits (Figure 1.20). Enteric *E.coli* strains are classified based on the above criteria into 6 pathotypes: Enteropathogenic *E.coli* (EPEC), Enterohaemorrhagic *E.coli* (EHEC), Enteroinvasive *E.coli* (EIEC e.g. Shigella sp), Enteroaggregative *E.coli* (EAEC), Diffusely adherent *E.coli* (DAEC) and ETEC [206, 207]. Recently two further pathotypes have been characterised,, Shiga toxin (Stx) producing Enteroaggregative *E.coli* (STEAEC), which was responsible for the German *E.coli* crisis in 2011 [208] and the Crohn's disease associated non-diarrhoeagenic Adherent Invasive *E.coli* (AIEC) strain [209]. Only the diarrhoeagenic pathotypes will be discussed in further detail.

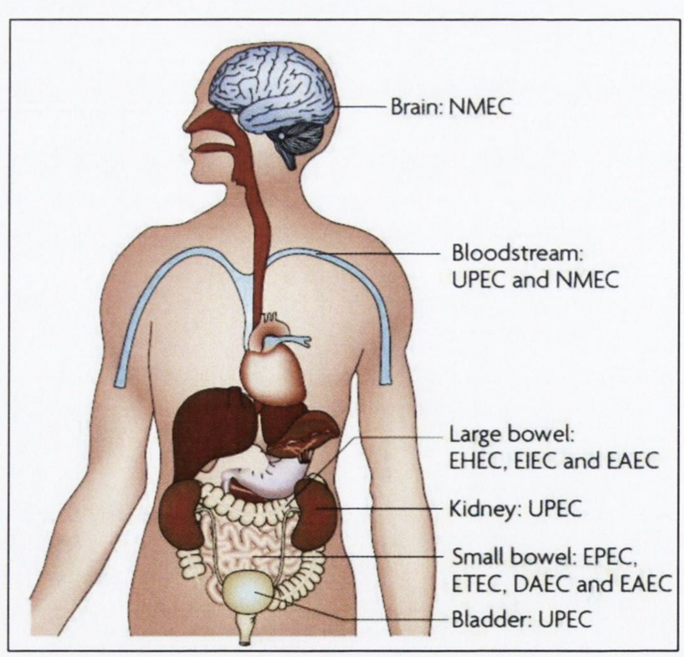


Figure 1.20 – The sites of entry and colonization by pathogenic E.coli. Pathogenic E.coli can invade the body through numerous routes and colonize distinct tissues. Depending on the strain and the tissue it infects different clinical manifestations of disease are encountered. (Taken from Croxen, M.A. et al, 2010)

1.13.2 – *Vibrio Cholerae*

Cholera is a severe Diarrhoeal disease caused by the enteric, non-invasive pathogen *V. cholerae*. This pathogen is widely dispersed, occupying many endemic areas mostly in developing countries which lack proper sanitation systems. Cholera also disseminates rapidly during natural and man-made catastrophes as epidemics but also as seasonal outbreaks (such as during rainy seasons). Despite the historical significance of Cholera in the western world, and indeed Ireland (Figure 1.21), it still remains an important global health concern. The most pronounced disease symptom is a profuse and rapidly dehydrating Diarrhoea. The disease is caused predominately by the O1 and O139 serotypes of the bacterium *V. cholerae* which is spread through contaminated water and food. Without appropriate treatment fatality due to the infection is common while with appropriate rehydration therapy this outcome is reduced to less than 1% [210]. Regardless of the circumstances under which cholera outbreaks occur they are the cause of many deaths in the susceptible groups, the group most at risk being children. Although cholera is both preventable through the provision of proper sanitation, clean drinking water, treatable with antibiotics and oral rehydration therapy, it still remains a major health problem in affected areas where various socio-economic limitations make the provision of these measures difficult. It is estimated that 3-5 million cases of cholera occur each year resulting in more than 100,000 deaths annually [211]. It is generally accepted that vaccines against cholera have the ability to play an important role as a public health measure for the prevention of cholera and other enteric diseases [212]. However, in order to provide the greatest benefit to public health these vaccines must be effective, widely available and affordable in lower income countries [212].

Figure 1.21 – Cholera epidemics took their toll on the populations of Dublin's tenement flats during the 1800s. A mass cholera grave can be found in Glasnevin Cemetery.

1.13.3 – Cholera and ETEC share a similar mechanism of pathogenicity

While V.cholerae and ETEC are distinct bacterial species they share a remarkable homology in how they exert their pathogenic effects. This effect is a result of the secretion of the heat-labile toxins CT and LT respectively whose adjuvant functions were introduced in section 1.11.1. These toxins share a remarkable sequence homology of over 80%, and so it is not surprising that they act by an almost identical mechanism (Figure 1.23) [134]. The toxins belong to the family of heterohexameric AB5 toxins (Figure 1.21 a). These are composed of an A subunit linked to CTB (Figure 1.21 a). The A subunit is composed of two parts, the enzymatically active A1 domain (hence "A" subunit for active) and the A2 domain with anchors the A1 unit (Figure 1.21 b) to the pentameric CTB subunit ("B" for binding) (Figure 1.21 c) [213, 214]. However, the mechanism by which these toxins are delivered to intestinal epithelial cells is different for CT and LT. While the CT holotoxin is secreted with remarkable efficiency, ETEC delivers LT via a system dependent on intimate contact between the bacterium and the IEC (the respective toxin delivery mechanisms will be reviewed later see section 1.43.3 for ETEC and 1.17.2 for *V. cholerae*).

After release from the bacterium, and upon encountering an IEC, the toxin molecule binds to the monosialoganglioside receptor GM1, which is centred in caveolae [215] that are present on all nucleated eukaryotic cells, via the pentameric B subunit [216, 217]. The outer-membrane vesicle is then internalized at lipid rafts and the LT transported into the cytosol via the endoplasmic reticulum (ER) in a Golgi-mediated transport pathway [218]. After internalization, CT is transported to the ER via a retrograde transport pathway, possibly via the Golgi apparatus [219], although the precise mechanism of intracellular transport into the cytosol for both LT and CT is not completely understood [213]. However, following its arrival in the ER, CTA dislocates from CTB and is transported to the cytosol, possibly by engaging pathways that transport misfolded ER proteins to the cytosol for degradation [220].

However, CTA escapes proteolytic degradation due to its low lysine levels, which are a target for ubiqunination (Figure 1.23). The toxicity of CT and LT is primarily exerted by the A1 subunit in the cytosol where CTA and LTA catalyse the ADPribosylation of the trimeric mammalian Gsα of adenylate cyclase, constitutively maintaining it in a guanine triphosphate (GTP)-bound and active state, thus inhibiting the GTPase activity of the enzyme [213] and resulting in unregulated levels of cAMP inside the cell [135]. The elevated intracellular concentration of cAMP leads to the phosphorylation of the cystic fibrosis transmembrane regulator (CFTR) chloride channel by the cAMP-dependent protein kinase A (PKA) [208]. This reaction leads to an imbalance of electrolytes in the cell, mostly a decrease in sodium uptake and an increase in chloride ion extrusion via the CTFR [221]. This leads to the secretion of electrolytes and water [222]. Interestingly subversion of the activation of PKA and other cellular pathways by LT was also shown to inhibit the expression of antimicrobial peptides [223], an important component of the intestinal mucosal immune system. LT has also been linked to the enhancement of bacterial adherence to intestinal epithelial cells [224], which in turn promotes the colonization of the small intestine [225] thus enhancing

pathogenicity. Ultimately however, the rapid secretion of water from cells into the gut lumen, which in extreme cases can be up to 2L per hour, leads to profuse osmotic diarrhoea [213].

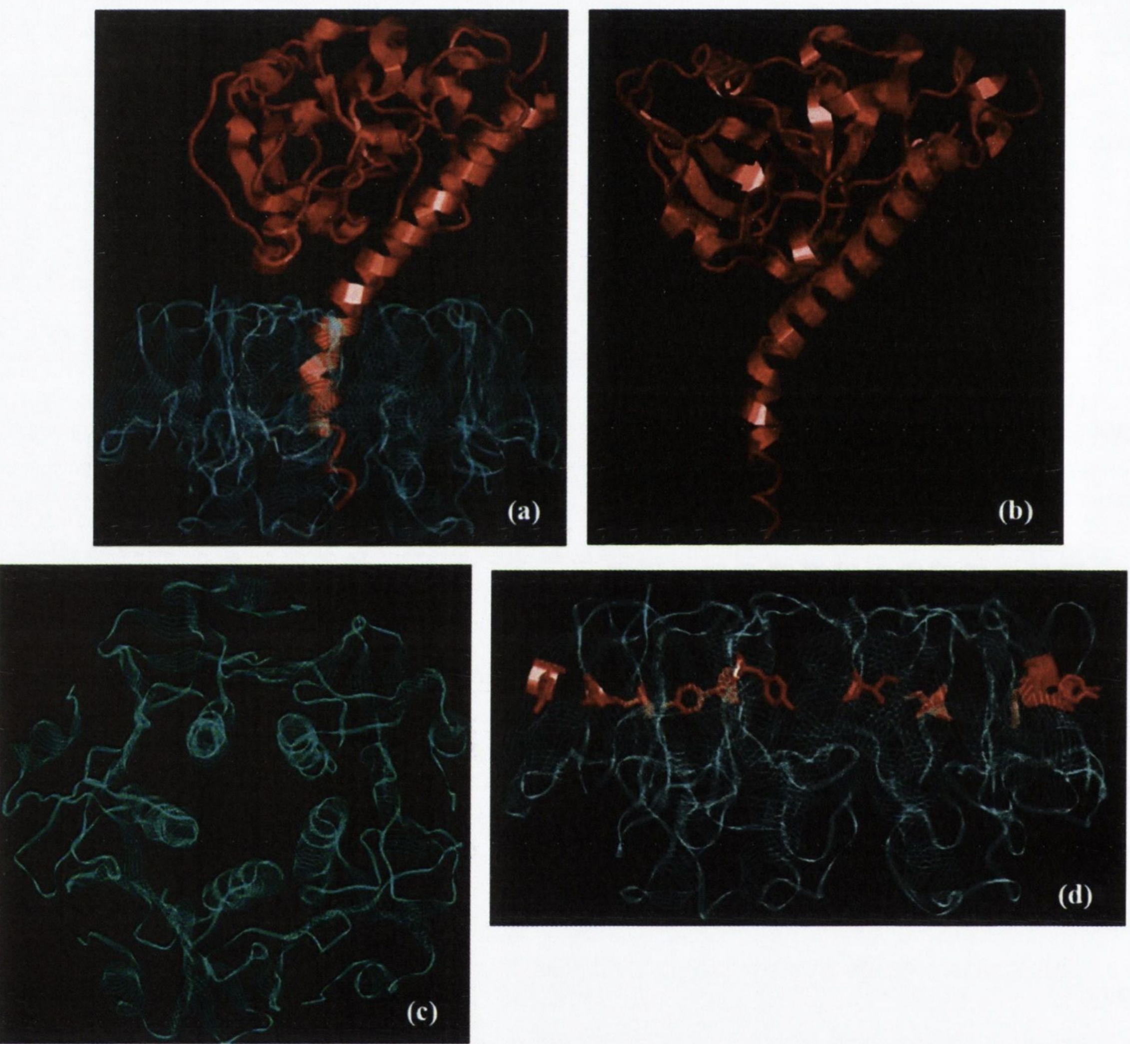


Figure 1.22 – The crystallographic structure of cholera toxin. CT holotoxin (a) is composed of an ADPriboylating A1 subunit (b), connected to a pentameric B subunit (c) by a helical A2 chain (d). The different position of residues in the CTB subunit between Classical and El Tor CTs is highlighted in red in (d). (Taken from Sanchez, J. 2011)

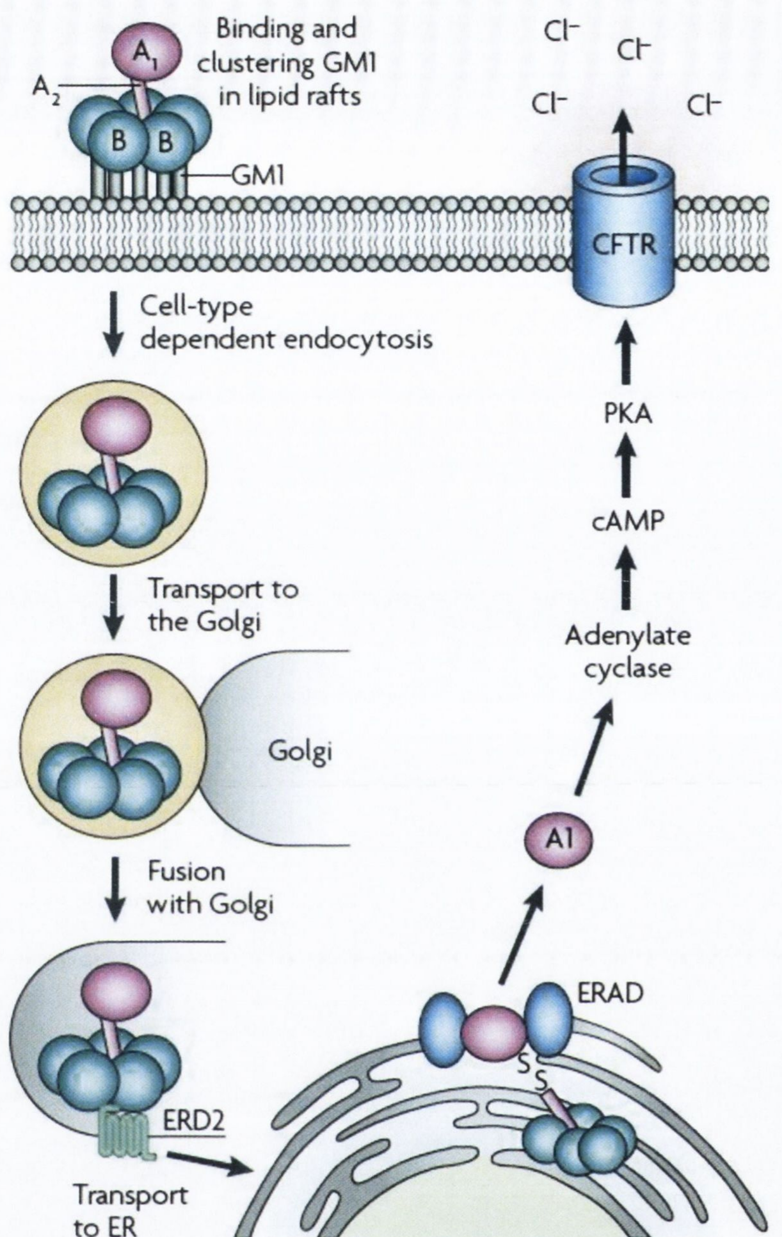


Figure 1.23 – The retrograde trafficking and disease mechanism of CT and LT. Both toxins enter a cell via the B subunit binding to GM1. After entry, the toxin is routed in a retrograde manner into the endoplasmic reticulum (ER) via the Golgi apparatus. A specific amino acid sequence, KDEL, located on the A2 subunit mirrors a host sequence that retains proteins inside the ER. A shuttle protein called ERD2 translocates the toxin from the Golgi into the ER. From the ER the toxin co-opts the ER-associated degradation (ERAD) pathway and usurps this to gain entry into the cytosol. Under normal conditions ERAD targets misfolded proteins in the ER for degradation in the cytosol. The toxin mimics a misfolded protein and enters the cytosol, but the A1 subunit escapes degradation after which it ADP-ribosylates adenylate cyclase leading to high levels of cytosolic cyclic AMP (cAMP) production. These elevated levels of cAMP activate protein kinase A (PKA) which then phosphorylates the cystic fibrosis transmembrane regulator (CFTR), leading to Cl- secretion out of the cell and into the intestinal lumen. (Taken from Viswanathan, V.K. et al, 2008)

1.14 – Epidemiology of diarrhoeagenic enteric E.coli pathotypes

Infections from pathogenic *E.coli* most often occur as a result of poor hygiene such as underdeveloped sewage systems or the consumption of contaminated food and fluids. Undercooked meat, contaminated fresh produce (e.g. Vegetables) and drinking water contaminated with human or animal wastes are the most common causes of infection [226, 227]. However, difficulty in obtaining accurate statistics on the incidence of enteric *E.coli* infections globally has led to unreliable statistics, as often the causative agent is not identified and many of these infections occur in endemic areas that suffer from poor development and health facilities (Figure 1.24). In developing nations the most prominent *E.coli* related causes of infant diarrhoea are ETEC, EPEC and EAEC often with fatal or developmental consequences. In the developed world, where better healthcare is available, these pathogens generally only cause mild and self-limiting infections ((Figure 1.24). EHEC, and more recently EAEC and STEAEC have been the primary pathotypes of E.coli associated with incidents of food poisoning in developed nations. Traveller's diarrhoea, often known as the "Delhi Belly" is a diarrhoeal illness that affects travellers in endemic regions of *E.coli* pathovars (Figure 1.25). Military personnel and foreign aid workers stationed in endemic regions often suffer from ETEC infections [228]. Although rarely fatal, this illness often causes severe discomfort to patients. ETEC (10-60%) [229, 230] is the most commonly isolated pathotype. So widespread is the disease burden of ETEC, it is suggested that over 10 million cases can be attributed to the pathogen alone annually [231]. In endemic regions, ETEC isolates from children in developing countries account for approximately 20% of cases, leading to hundreds of millions of infections and tens of thousands of deaths per year, and is the most frequently identified bacterial enteropathogen [232].

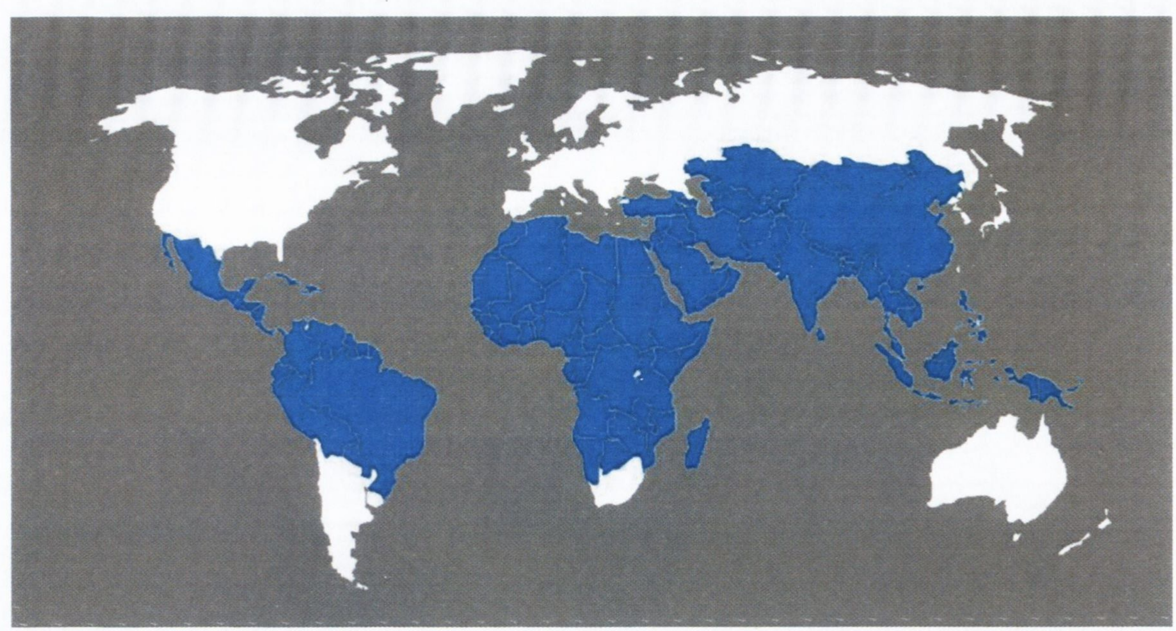


Figure 1.24 – The global burden of ETEC extends to over 50% of the world's population. (Source CDC, 2007)

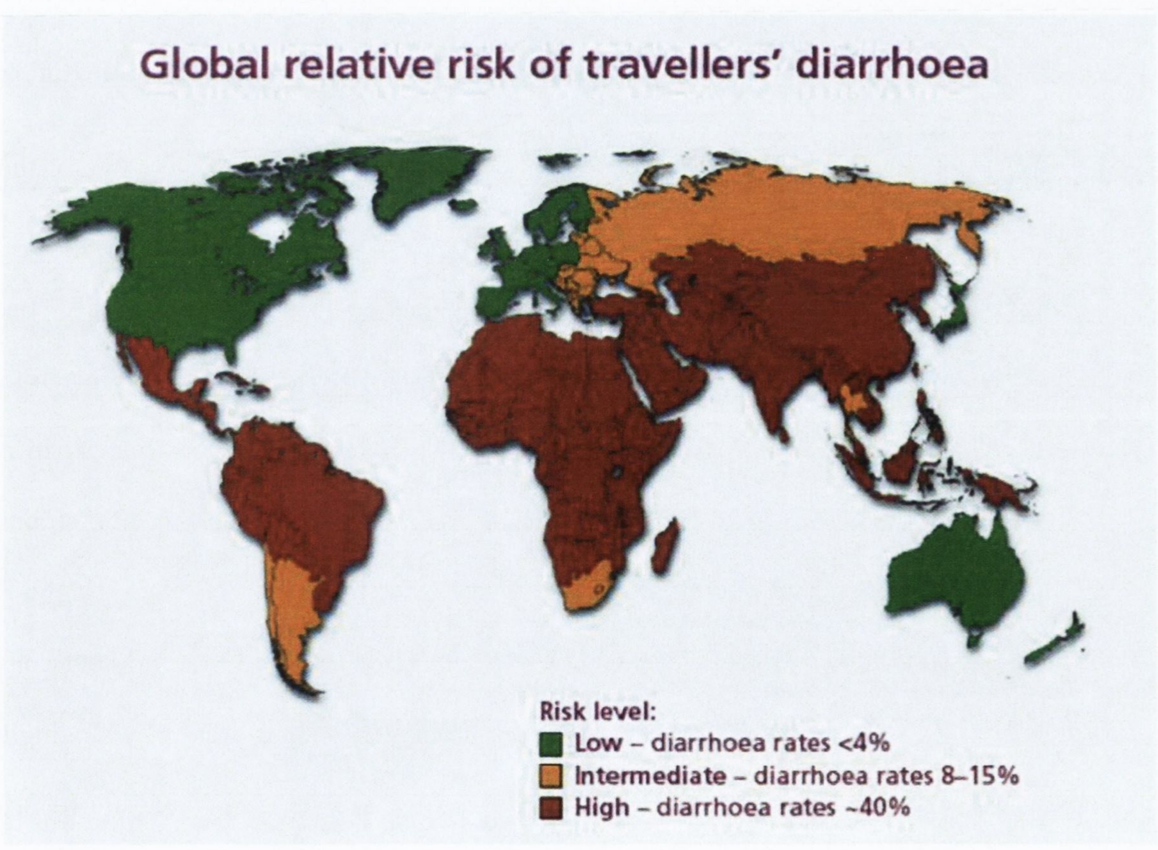


Figure 1.25 – The global risk of Traveller's Diarrhoea is extremely high. (Source Health protection agency, UK, 2010)

1.14.1 – ETEC; from a friend to a foe

ETEC is one of the most prevalent enteric *E.coli* pathotypes causing hundreds of millions of diarrhoea cases annually, most seriously 300,000-500,000 cases in children under 5 in the developing world [233]. Despite its impact on global health, ETEC is one of the least complex pathotypes to emerge from the enteric *E.coli* family. ETEC is not an invasive pathogen and does not employ a secretion system to directly translocate bacterial proteins into target cells in order to subvert physiological processes (unlike EHEC, EPEC and EIEC which require this system for virulence). Instead of relying on this complex system, ETEC has evolved a much simpler and highly efficient process to cause virulence. A combination of effective and selective colonization factors (CFs) and powerful secreted toxins provide ETEC with the array of molecular tools it requires to exert its virulent effects (Figure 1.26).

1.14.2 – Colonization factors of ETEC and adhesion to intestinal epithelial cells

CFs are a heterogeneous group of proteinecious surface structures, and were one of the first virulence factors discovered in ETEC [234]. Currently over 25 distinct CFs have been identified in different strains of ETEC [235], which mediate the adherence of ETEC bacteria to intestinal epithelial cells (Figure 1.26). These CFs can differ in length; ranging from 1 to greater than 20 μm and structure; being fimbrial, non-fimbrial, helical or fibrillar in nature [236]. It is the acquisition of CFs rather than the emergence of species-specific strains that allows ETEC to colonize different hosts, such as cattle and piglets [237]. Interestingly, ETEC has not just evolved appendages for attachment to epithelial cells, but also to other cells that will aid its transmission. ETEC has developed the ability to attach to leaves by binding to the leaf surface via its flagellar staff [238]. However, 30-50% of ETEC isolates are reported to possess no characterized CFs [232], new ones are constantly being identified and categorized [239]. Despite the large repertoire of colonisation factor antigens (CFAs) already detected on

ETEC strains, CFA/I, II and IV the most common [240]. The cognate receptors for CFs have yet to be identified, CFA/I is however thought to bind to carbohydrate moieties of non-acid glycosphingolipids and glycoproteins [241]. Recently EtpA, a selective adhesin located on the tip of the flagella, was shown to mediate the transient attachment to mammalian intestinal cells [242]. EtpA is eventually degraded by the serine protease autotransporter of Enterobacteriaceae, EatA. This process both modulates bacterial adhesion and potentiates the delivery of LT into the cell to which ETEC is attached (Figure 1.26) [243]. While both CFs and flagella act as anchors for the initial attachment to epithelial cells, other outer membrane proteins serve to mediate the more proximal attachment (Figure 1.26). Two further proteins Tia and TibA, a glycosylated autotransporter, have been shown to mediate the intimate attachment of the bacteria to epithelial cells [244]. Interestingly, the functions of ETEC adhesion molecules are not limited to cell attachment but they also play a significant role in the delivery of various toxins to the host cells and thus play a key role in virulence.

1.14.3 – Toxin secretion by ETEC is the primary cause of pathology

Pathology caused by ETEC is a result of the secretion of plasmid-encoded toxins that subvert normal cellular processes. ETEC bacteria secrete two toxins, LT or heat- stable toxin (ST). These toxins are delivered through a secretion pathway, as opposed to the initial theory of release by bacterial lysis (Figure 1.26). However, the uptake of the toxin is target-cell mediated and ETEC does not employ and endogenous translocation system. However, unlike CT which is secreted and then taken up by epithelial cells, LT remains membrane associated by binding to LPS [245] and is highly dependent on bacterial adhesion to epithelial cells to ensure optimal delivery [246]. This requirement for bacterial adhesion to epithelial cells is evolutionarily highly beneficial as anti-LT antibodies can quickly bind to and neutralize free LT. Intimate contact with the target enables the ETEC bacteria to shield the LT [247] which is secreted in outer membrane vesicles (OMVs) rather than being secreted unshielded into the

intestinal lumen (Figure 1.26). Studies have shown that LT and its secretion apparatus can cluster to one location on the bacterial outer membrane, thus delivering the toxin payload in a highly specific and concentrated manner, ensuring maximum penetration into target cells [248]. The mode of action of LT was outlined earlier in section 1.13.3 (Figure 1.23).

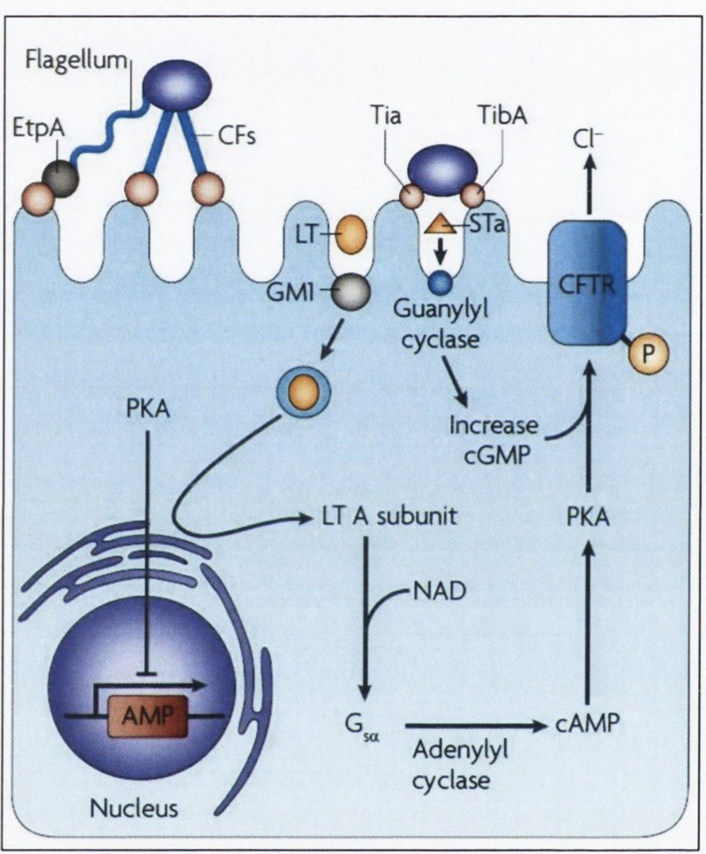


Figure 1.26 – The mechanisms of pathogenicity of ETEC. ETEC becomes anchored to IECs in the small intestine through CFs and EtpA, an adhesion molecule found at the tip of the flagella. Intimate contact is mediated through Tia and TibA. Toxicity is mediated through two toxins, LT and ST which are both secreted into the cell and result in Diarrhoea through cAMP and cGMP-mediated activation of cystic fibrosis transmembrane regulator (CFTR) leading to the excretion of chloride ions into the gut lumen and this ion imbalance results in Diarrhoea. (Taken from Croxen, M.A. et al, 2010)

ST toxin is a cysteine-rich peptide molecular mimic that imitates the native intestinal hormone guanylin. Much smaller than LT, ST or STa the toxin associated with human disease, are synthesized as a 72 amino acid pro-form, which is then cleaved into its active, 18-19 amino acid active form. ST binds to and activates the guanylate-cyclase-C (GC-C) receptor located on the intestinal brush boarder (Figure 1.26). This initiates a signalling cascade that leads to the elevation of intracellular messenger cyclic GMP (cGMP) concentrations. cGMP activates protein kinase II which results in the phosphorylation of CFTR thus driving Cl^- secretion and impaired NaCl absorbtion [249-252]. LT and ST act synergistically to elicit electrolyte secretion by the intestinal epithelial cells into the lumen of the intestine which results in fluid accumulation and osmotic Diarrhoea.

1.14.4 – ETEC and Clinical manifestations of infection

Apart from Diarrhoea, ETEC contributes to the delayed growth and malnutrition of children suffering repeated bouts of infection (section 1.13). Indeed it is a vicious cycle as malnourished children display a higher risk of contracting ETEC infection [203, 253]. ETEC infections can range from asymptomatic to a severe and profuse watery diarrhoea [222]. This results in rapid dehydration and prostration within hours [254]. Infections are often mistaken for cholera as these cannot be distinguished on the basis of clinical presentation [255]. ETEC infections can also cause nausea, fever and vomiting, with some patients suffering prolonged diarrhoeal episodes which can last over a week [256].

1.15 – Vaccine strategies against ETEC

No ETEC vaccine has been licensed for medical use, although scientific evidence has strongly supported the development of such a vaccine. Studies have highlighted the potential of CFs as candidate vaccine antigens [257], despite many ETEC strains not expressing recognizable CFs. A CF-based vaccine induced protection against severe diarrhoea from CF-homologous

strains in travellers to Guatemala but this vaccine failed to elicit protection in children from Egypt [257, 258]. A study in Bangladesh found that exposure to ETEC prevents against subsequent infection and diarrhoea from ETEC of the same CF type [253]. Similarly, Egyptian children with detectable anti-CFA/I antibodies displayed an inverse correlation with the subsequent incidence of diarrhoea caused by ETEC expressing homologous CFs. Interestingly these findings correlated with a trend in ETEC endemic regions where there is a decline in ETEC related diarrhoea in individuals as they get older. The peak of these diarrhoeal episodes is in infants aged 6-18 months, which also constitutes the highest demographic for ETEC related mortality in developing countries [232, 253]. This phenomenon is not observed in travellers who visit endemic areas for short periods of time [232]. However, the longer a non-indigenous person from a developed nation spends in an endemic area, the faster the frequency of incidences of ETEC induced diarrhoea declines as natural acquired immunity to the pathogen is established. These findings illustrate that over the course of repeated natural ETEC infections an individual can develop robust immunity to the pathogen which provides encouragement for the development of a mucosal vaccine. The various antigenic targets for ETEC vaccines will now be discussed.

1.15.1 – Toxins

The toxins produced by ETEC are regarded as key virulence factors [151, 232]. ST is a very poorly immunogenic peptide, possibly due to its small size [259]. Following infection of humans with ST producing ETEC bacteria, antigen-specific responses against ST are not detectable. Conversely LT and more specifically the B subunit of LT (LTB) is highly antigenic. Infection elicits toxin-specific mucosal and systemic immune responses. Interestingly there exists an 80% homology between LTB and CTB, which is also highly immunogenic [151]. However, when considering which antigen to include in a vaccine

formulation one must consider that ETEC can produce either ST or LT alone or both and strains vary greatly between different endemic regions [151, 232, 259].

1.15.2 – O-serogroups

The O-antigen is located on the tip of the LPS molecule, and is used to categorize serogroups. ETEC is extremely heterogeneous and there are more than 100 different identifiable pathogenic serogroups [260]. Even though some o-antigens have a high prevalence, their global distribution is so diverse, making an o-antigen-based oral ETEC vaccine (OEV) an inefficacious choice for a pan-regional vaccine.

1.15.3 – Colonisation factors

As discussed previously ETEC bacteria express several types of appendage which determine host specificity, adhesion, colonization and toxin delivery, making them important virulence factors, with 25 of these having been identified in clinical ETEC isolates [235, 261]. Despite the large number of identified CFs, some are more prevalent in clinical samples than others including CFA/I, CS1, CS2, CS3, CS4, CS5 and CS6. Combinations of these CF molecules may also be expressed on particular strains of ETEC and CF expression can vary greatly between regions, seasons and patient demographics [232]. Several of the CFA/I- like molecules have been shown to be related, specifically CFA/I, CS1, CS2, CS4, CS14, CS17 and CS22. It has been shown that ETEC infection induces robust CF-specific immunity against not only the CF expressed on the strain but also against other related strains in the respective group [232]. This could be attributed to the structure of the CFs which are typically composed of a large number of identical structural subunits with several CFs possessing a distinct protein motif on their tips accounting for the specificity of certain CF antigens [261].

1.16 – Development of an efficacious oral ETEC vaccine

The relative success of Dukoral®, the currently licensed oral cholera vaccine (OCV) has prompted much research into the development of an OEV. Initial experiments showed that the administration of antisera against LT and purified E.coli LPS provided protection against challenge with the cognate LT-producing O group ETEC strains [262]. Similarly anti-CFA/I sera with or without anti-LT sera provided protection against CFA/I positive LT-producing strains, with a synergistic effect observed with co- administration of anti-CFA/I and anti-LT sera [262]. The identification of CFs as a possible key antigen target led to experiments to determine the effectiveness of blocking CF-mediated adhesion by ETEC. Mixtures of ETEC bacteria were first incubated with anti-CF monoclonal antibodies prior to challenge. The results of these studies showed that anti-CF antibodies interfered with bacterial pathogenicity [263]. Furthermore, infection of rabbits with ETEC strains expressing particular CFs resulted in significant protection against reinfection with homologous strains, but not heterogeneous ones [263]. Interestingly, these results also corroborate a study in Bangladesh showing that reinfection with CF expressing homologous strains is rare, but this is not evident in LT-producing strains where subsequent infections are common [253], possibly due to the actions of other attachment factors.

1.16.1 – An oral inactivated ETEC vaccine; is it a feasible solution?

Infection with ETEC bacteria expressing particular CFs induced protection against subsequent reinfection with homologous or related CF expressing ETEC strains. However, due to safety concerns it is not possible or desirable to vaccinate with live or attenuated ETEC strains. Formalin inactivation was shown to kill the bacteria without degrading the structural integrity and antigenicity of the CF molecules on the outer membranes [259]. WCK are preferred as they are more resistant to degradation in the GIT than purified CFs or subunit antigens. This

may be in part due to the structure of the CFs being preserved by formalin treatment and it is still possible for these dead bacteria to bind to epithelial cells and to be taken up by APCs (Joshua Tobias, *Personal Communication*). Furthermore, delivery of the vaccine orally has the potential to elicit immunity in the small intestine. In order to enhance immunity to LT-producing ETEC strains, and develop a bivalent anti-toxic and anti-bacterial vaccine, formalin killed bacteria can be combined with a modified LT toxoid or LTB [259]. Not only would bivalent protection be advantageous, but the synergistic effect observed between CFA/I and LT antisera would suggest that enhanced protective immune response using both antigens is possible [262]. This beneficial effect may reflect the ability of CTB and thus perhaps LTB to promote IgA producing B cells [141], which are important for vaccine elicited protection from ETEC.

With these criteria in mind, the research group of Ann-Marie Svennerholm in Gothenburg, Sweden developed such a bivalent OEV. Learning form the successes of Dukoral®, the group co-formulated an OEV consisting of formalin killed CFA/I and CS1-CS5 expressing ETEC bacteria, which also expressed prevalent O-antigens together with recombinant CTB (rCTB) which as stated before has a high structural similarity with LTB. In Swedish volunteers this rCTB-CF OEV elicited strong local IgA responses in 70-90% of subjects [264]. Results from Phase I and II trials in adult subjects from Sweden, Egypt and Bangladesh indicated that the vaccine was well tolerated and effective at driving mucosal immune responses (70-100%) against the various CFs present in the OEV [232, 265, 266]. The study was repeated in children in developing countries to assess safety and efficacy and was shown to be well tolerated and equally as immunogenic in children as in adults [259, 267]. However, in infants aged 6 to 17 months vomiting was observed. In order to overcome this a dose ranging study was initiated to identify a safe dose, which resulted in immune responses against both LT and CFs in the OEV [268]. In later phase III trials of the rCTB-CF OEV protective efficacy was

measured in American travellers in Mexico and Guatemala. Subjects vaccinated with the rCTB-CF OEV were significantly better protected (77%) against non-mild ETEC diarrhoeal illness, defined as symptoms that did not interfere with the daily routines of the travellers [258]. The same vaccine was trailed in rural Egypt with children 6-18 months old. Protection induced by the vaccine in this study was very poor (20%). However, most cases of ETEC diarrhoea encountered in this study were very mild in comparison to unvaccinated individuals. This may have reduced the protective scoring of vaccinated individuals by not accounting for the reduced severity of disease. More importantly, it was noted that the immune responses elicited in children in this study were much lower than seen in older children in the same setting [257]. These results are however consistent with a trend that an oral vaccine often perform less effectively in young children and infants in developing nations when compared to adults in developed nations [269]. Preliminary work in rodents implicates a deficiency of vitamin A as a result of malnutrition, in the reduced capacity to mount a mucosal immune response [270]. Such deficiencies are well documented in developing countries. In importance of the vitamin A metabolite RA and its role in IgA induction in the gut was described earlier.

1.17 – Cholera Epidemiology and recent outbreaks

The epidemiology of cholera was one of the first diseases in history to be studied in this manner. Although more than 200 *V. cholerae* serogroups have been identified, approximately 98% of global disease is caused by O1 while a small percentage of cases have been reported due to the O139 serogroup which is found in Asia [271]. Two biotypes of pathogenic *V. cholerae* have been characterized; El tor and classical which can be further subdivided into 2 serotypes Inaba and Ogawa. New variant strains of El tor have been reported in Asia and Africa which express a toxin similar to the classical biotype but cause a more severe infection [272]. Furthermore, an increase in the number of strains displaying antibiotic resistance is a growing concern, which complicates treatment [272]. The world health organisation (WHO)

reported over 221,226 cases of cholera in 2009 which culminated in 4946 deaths across 45 countries. Moreover, 98% of these cases and 99% of deaths were reported in African countries [273]. However cholera cases are notoriously under- reported globally and it has been reported that approximately 3 million cases of cholera occur annually in Asia resulting in 120,000 deaths [274]. Young children and infants are most at risk of cholera infection, but the disease can affect humans of any age, especially in endemic locations [275].

While people in endemic locations are exposed to a constant cholera threat, the devastation caused by this disease during an outbreak is truly astonishing. Cholera outbreaks most often occur in the wake of natural disasters and war. During the Rwandan conflict of the early 1990s, millions of Rwandan refugees fled their country in the aftermath of genocide to the great lakes region of Zaire. Displaced and with no homes to go to these people set up enormous refugee camps most notably near Goma. A complete lack of sanitation, clean water and medical facilities soon led to a cholera outbreak which led to over 70,000 cases and 12,000 deaths [276].

Another devastating cholera outbreak occurred in Zimbabwe in 2008/2009. Prior to the election of Robert Mugabe in 2008 to the role of president Zimbabwe had one of the best healthcare and sanitation systems of any African nation. However, political unrest in the wake of Mugabe's appointment led to a breakdown in healthcare and sanitation which culminated in a cholera outbreak. The impact of this outbreak was so great that it accounted for 30% of global cholera cases recorded that year [273]. Further outbreaks in 2010 were responsible for 40,000 cases and approximately 2000 deaths across Cameroon, Chad, Niger, Nigeria and Pakistan [277]. In the wake of the Haitian earthquake, which devastated the world's poorest nation in 2010 a cholera epidemic further complicated the already fragile disaster situation. By January 16th 2011 over 194,000 cases of infection and 3819 deaths were contributed towards global cholera disease statistics by Haiti alone [278]. Incidents of cholera disease

tend to increase during periods of warmer temperature. More and more studies are displaying a correlation between the rise in the global burden of cholera and global warming due to climate change [279].

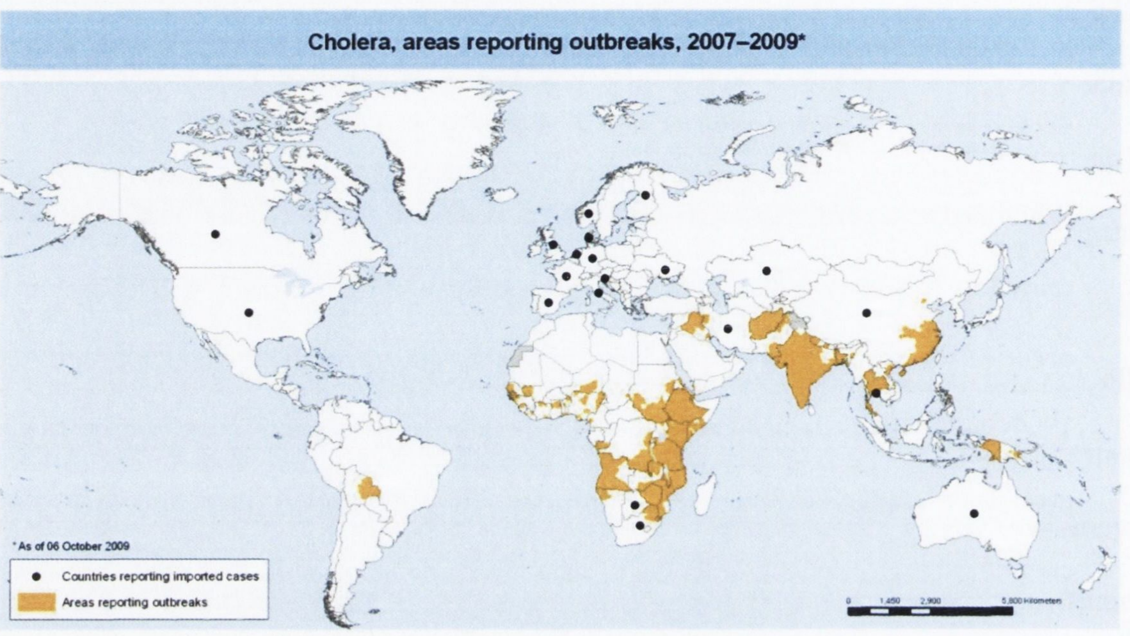


Figure 1.27 – Cholera outbreaks 2007-2009. (Source WHO, 2009)

1.17.1 – Clinical Pathophysiology and Virulence

The first step of cholera infection is the colonization of the small intestine by *V. cholerae* vibrios. The pathogenicity of *V. cholerae* is primarily as a result of CT. Following release, the combined actions of both subunits leads to the uptake of CT by IECs, the subversion of the host cell machinery and the secretion of ions into the gut lumen which results in rapid fluid accumulation due to the osmotic imbalance (Figure 1.26). This results in the secretion of both water and electrolytes in a profuse and watery diarrhoea which can result in rapid dehydration and death [151].

1.17.2 – Cholera Toxin, the good, the bad, the ugly

CT is a very interesting molecule due to its Dr. Jekyll and Mr. Hyde characteristics. As a research tool CT has been invaluable, both in the development of new mucosal adjuvants and as a means to dissect various mucosal immune system mechanisms. Its GM1 binding and retrograde trafficking ability have also been exploited to visualize cellular processes in many cell types. While the virtues of CT are apparent to the research community not all would regard it with such fondness. The specific role of CT as the active cause of cholera was proposed by Koch in 1884 where he referred to it as a "poison" of *V. cholerae*. The role of CT in pathogenesis was introduced in 1.13.3 while its immunomodulatory role as an adjuvant was discussed in 1.11.1. While over 140 *V. cholerae* serogroups exist, few have the ability to produce CT and cause disease, most noticeably the O1 group. The O1 serogroup can be further subdivided in Classical and El Tor biotypes which express slightly different CT molecules. While the CTA subunits are identical, there are subtle amino acid differences in CTB [280]. Although these residues do not take part in Gm1 binding, they are presumed to be part of an anti-CTB bio-type specific monoclonal antibodies and so may represent an evolution of the pathogen to evade host immunity [281]. Expression of CT is not constitutive, rather it is inducible. External influences such as "quorum-sensing" whereby the pathogen turns on CT expression when surrounding *V.cholerae* numbers are low and off when they are high, external cAMP concentration, pH and bile acids can all indicate to the organism when it has reached the small intestine [282]. Unlike LT, which is secreted after intimate contact is made with the intestinal epithelium by ETEC, CT is secreted into the intestinal lumen by *V. cholerae* [213]. More than 90% of CT is usually found extracellularly in its soluble form, instead of being held intra-cellularily by the bacterium, demonstrating the incredible efficiency the organism has developed to ensure pathogenicity [283].

1.18 – Vaccines against Cholera

Injectable WCK cholera vaccines, dating back to Louis Pasteur in the 19th Century, were used up until the 1970s. However, due to poor safety standards, unfavourable and low durations of efficacy these have been phased out and are not recommended for use anymore [284]. The 1970s also saw an explosion in the interest in the disease which culminated in not only a better understanding of the bacterium and the disease pathway but also of the immune response that leads to protection. Thus efforts to elicit protection by parenteral vaccination were abandoned in favour of those that stimulated local mucosal secretion of antibodies in the intestine. Furthermore, it was determined that the oral administration of antigens is the most efficient method of eliciting mucosal immune responses in the case of cholera [285].

1.18.1 – Antigenic targets of V.cholerae

Currently all three OCV formulations contain both variants of the different serotypes (Inaba and Ogawa) and biotype (El Tor and classical) of the O1 strain of *V. cholerae*. Both Shanchol™ and Orc-Vax™ also contain the O139 serotype which emerged from various cholera epidemics in the 1990s. However as this strain represents only a tiny fraction of cholera cases each year (<1%) most vaccine efforts are focused on generating vaccines against the O1 strain and its variants. While the Inaba strain of the El Tor biotype is thought to be the cause of all modern cholera outbreaks across the globe [286], two other strains, one Inaba and one Ogawa, both of the classical biotype where thought to have been the cause of all pandemics from the 1800s up to the 1960s [286]. While genetically quite similar both strains have very different biochemical characteristics [286].

The difference between serotypes is the presence of a methyl group on the terminal persoamine on the tip of the surface LPS molecule in Ogawa O1 *V. cholerae* which is not present on the LPS of the Inaba variants [287]. The selection between serotypes is controlled

by the webt gene, which codes for an adenosylmethionine-dependent methyl transferase and when mutations deactivate this gene, the Inaba phenotype results. The *webt* gene thus accounts for the three different antigenic variants of the LPS on O1 *V. cholerae*. The polyperosamine A antigen is shared across all O1 variants, the B antigen results from the methylation of the terminal persoamine on the LPS of the Ogawa strain and the C antigen of Inaba LPS which lacks this methylation on the terminal persoamine [287].

Humans are capable of mounting both a local intestinal mucosal immune response to cholera as well as a systemic one. This immune response to challenge or infection with *V. cholerae* vibrios is capable of eliciting protection against subsequent re-infection with the same serogroup [288]. Although protection against CT can be elicited by infection with different serogroups, no cross-protection against heterogeneous strains has been recorded [289]. Serum vibrocidal antibody titres from individuals native to endemic regions have been shown to increase with age and these are inversely related to the susceptibility to developing the disease [290]. These results have led to the use of serum vibrocidal antibody titres as a marker of the immune response to cholera vaccines; however the significance of these antibodies in the defence against cholera is debated [285].

1.18.2 – Current vaccine strategies against cholera

There are three predominantly employed strategies for generating effective OCVs. WCK vaccines, subunit vaccines and genetically attenuated live vaccines [285]. To date the only successfully licenced oral vaccines against cholera are in the form of WCK formulations. These are found to be the most effective formulations at stimulating intestinal mucosal immune responses, which have been found to be critical to protection against cholera infection [291].

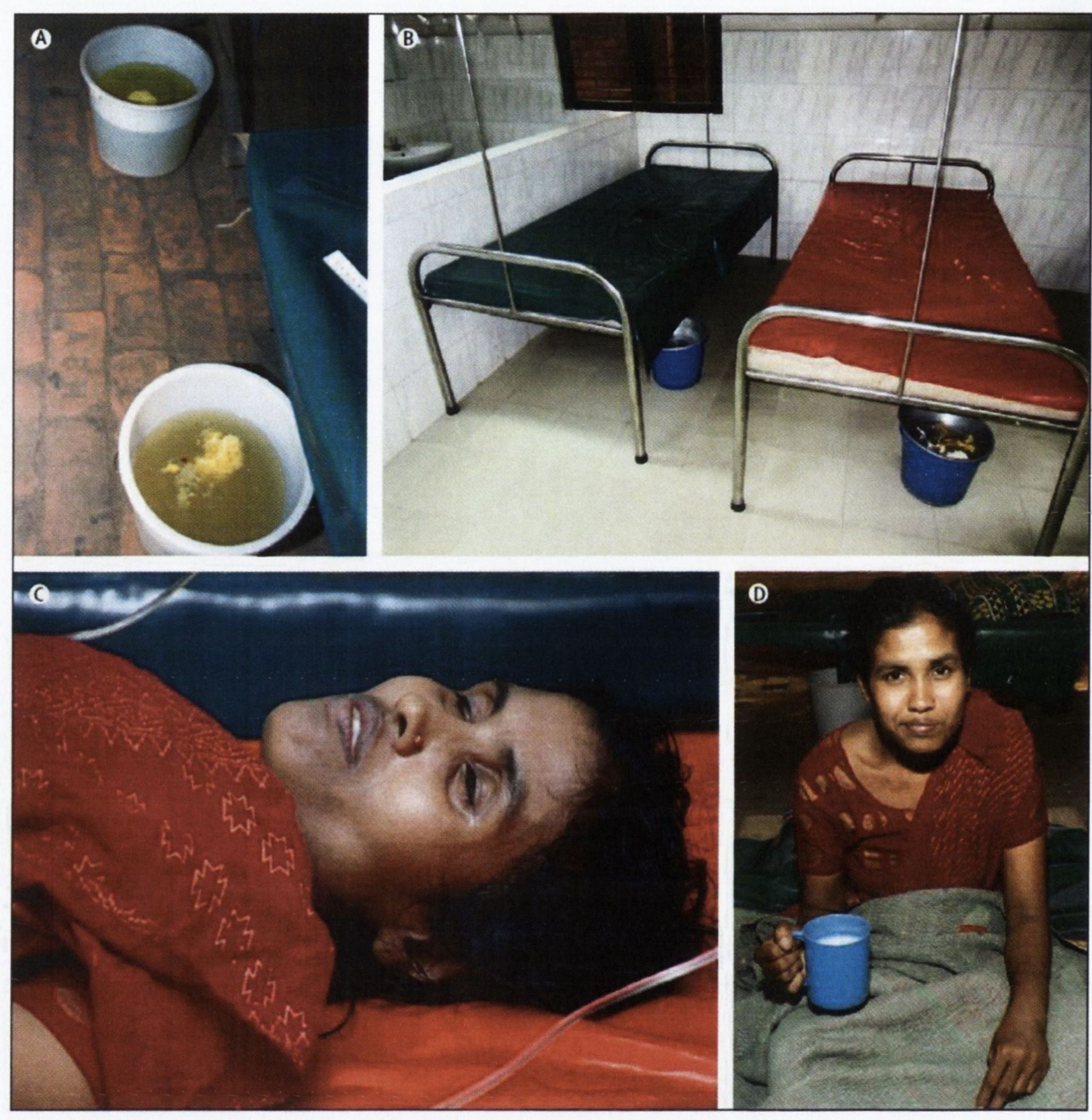


Figure 1.28 – Cholera management strategies. (a) Rice-water stool is the universal symptom of cholera. (b) "Cholera-beds" allow the safe evacuation of rice-water stool and the monitoring of the ablation of symptoms. (c) Patient with cholera shows signs of fatigue and dehydration. (d) Patient begins rehydration therapy, and after 8 hours shows dramatic signs of improvement.

1.18.3 – A Whole Cell killed vaccine containing the cholera toxin B subunit

In 1991 Crucell/SBL Vaccines introduced a novel OCV consisting of WCK *V. cholerae* supplemented with rCTB. Dukoral® was the first oral cholera vaccine to be developed and licensed. This vaccine provides bi-functional protection by generating both anti-toxic mucosal antibodies against CTB and anti-bacterial vibrocidal antibodies against *V. cholerae*. Dukoral® provides protection against CT for 6 to 9 months following administration, however, this protection subsides afterwards resulting in longer term protection against the *V. cholerae* bacteria only [292]. Aside from providing bi-functional protection, the inclusion of rCTB into whole cell killed cholera vaccines also provided protection against ETEC diarrhoea [293-295]. The WCK component of Dukoral® consists of a mixed suspension of both El tor and classical biotypes expressing Inaba and Ogawa serotypes of O1 *V. cholerae* together with rCTB. The vaccine is intended for use from ages 2 and upwards with adults and children 6 years or older requiring 2 doses and three doses from children under 6. As CTB is sensitive to the gastric environment the vaccine must be co-administered with a liquid buffer to neutralize stomach acid. The buffer is made up in clean water which is then mixed with the liquid vaccine which requires cold storage [285].

Dukoral® was the culmination of two previous oral vaccine efforts. Both vaccines included WCK *V. cholerae* bacteria but only one of the two was a WCK+CTB vaccine with the CTB being produced by chemical extraction of the B subunit from CT. The WCK+CTB was found to be both safe and well tolerated resulting in 85% protection for up to six months after administration, 62% after 1 year and 58% protection after 2 years in Bangladesh [296]. The vaccine also elicited sort-term protection (up to 6 months after vaccination) against LT producing ETEC strains [295]. Later refinement of the production methodologies employed to generate the vaccine led to the use of a recombinantly produced CTB from an engineered strain of *V. cholerae*. Trials using this formulation showed improved short term protection

(86%) being conferred on volunteers against El tor strains [297]. While a further trial failed to elicit strong protection, a 2-dose regime administered to children and adults in Mozambique conferred 82% and 84% protection respectively [298]. While Dukoral® has been accepted and licensed by the WHO, its use has been primarily for travellers to endemic regions and humanitarian/military personnel to outbreak regions due to the high cost of the vaccine, approximately €70 per dose (commercial retail price) [285].

1.18.4 – Whole cell vaccines lacking the B subunit of cholera toxin

As mentioned previously, the Bangladeshi trial also included a group of volunteers who received a WCK OCV lacking CTB. While this vaccine conferred modest (58%) short term protection (up to 6 months), protection was sustained considerably longer with 60% showing protection after 2 years and 42% after 3 [292]. These data encouraged the government of Vietnam to embark on its own project to develop a locally produced OCV based on WCK *V. cholerae* without CTB. Trials of the local vaccine showed that a 2-dose regime conferred 66% protection against cholera in individuals over 1 year of age for 8-10 months after vaccination [299]. Furthermore, the addition of WCK vibrios of the O139 strain led to the local producer VaBiotech licensing the vaccine as ORC-Vax™. Later trials revealed that protection against El tor cholera was maintained for 3-5 years after oral vaccination [300].

While this vaccine does not confer cross-protection against ETEC induced diarrhoea, it has other virtues making it a competitor to Dukoral®. Firstly as it does not contain rCTB, ORC-Vax™ is significantly easier to manufacture and cheaper. Secondly the vaccine does not need to be co- administered in a buffer, further reducing costs. 20 million doses of this vaccine have since been administered to Vietnamese vaccinees [300]. With the large scale efficacy of the Vietnamese OCV effort and the lower cost associated with this the International Vaccine Institute together with VaBiotech set about developing a modified ORC-Vax™ (mORC-

Vax^{TM}). This was undertaken as the production methods used by VaBiotech for the manufacture of ORC-Vax^{TM} were not adaptable to international good manufacturing practise (GMP), the standardization tests were not in line with WHO standards and the vaccine was also found to include traces of CT [285]. In order to address these shortcomings a new vaccine containing O1 and O139 serogroups was developed. The high-toxin producing strain was removed and replaced with 2 alternative Inaba and Ogawa expressing strains. Furthermore, the LPS content of the vaccine was doubled and modern quality control practices put in place to ensure amongst other standards, that CT was not present in the final formulation [301].

Field trials of this modified vaccine showed it to be both safe and immunogenic with sero-conversion rates of 91% against O1 V.cholerae in adults [302]. In vaccinees from Kolkata, an endemic region with high background immunity to *V. cholerae*, the vaccine elicited 52% protection in adults and 80% in children over 1 year old [303]. Later large scale phase III studies with adults and children in the Kolkata slums found that after 2 years 67% vaccinees were protected against El tor cholera [304]. Interestingly, protection in year 3 was reported at similar levels with the study currently on-going [285]. Following on from the results of these clinical trials, mORC-Vax^{TM} was licenced in Vietnam and went into production in 2009. However, as the regulatory authorities in Vietnam are not recognized by the WHO, the vaccine is also produced by Shantha Biotechnics in India who market the product internationally under the name Shanchol^{TM}, allowing the vaccine to be purchased by the United Nations.

1.18.5 – Challenges for oral cholera vaccines and experimental vaccines

Recent studies conducted by the WHO have recommended that oral cholera vaccination programs be established in areas where cholera is endemic and that strategies be put in place

in regions where cholera outbreaks are either on going or high risk [273]. However, achieving these goals is complicated by the manufacturing procedures used to make oral cholera vaccines. The three different *V. cholerae* bacterial strains must be grown and then inactivated by different methods thus requiring multiple batches of bacteria to be cultured. El Tor strains are inactivated using formalin while the classical Inaba strain is inactivated by heat treatment after which all whole cell killed strains are mixed together. The manufacturing process for Dukoral® further complicates this procedure as rCTB needs to be produced and purified from a specially engineered strain of *V. cholerae* under GMP conditions.

One final serological variant of O1 *V. cholerae* is the Hikojima strain which expresses both the Ogawa and Inaba LPS epitopes. This rare phenotype has been suggested to be a transition state between Ogawa and Inaba as many Hikojima strains are subsequently typed as Inaba, however, some stable strains of Hikojima do exist [305, 306]. Although the molecular basis for this novel phenotype has not been fully resolved, it is thought that the webt gene is attenuated rather than deactivated which alters the expression of LPS to include both the Inaba and Ogawa antigen on the surface of the same bacterial cell.

Rationalizing the formulation of an OCV using a simplified manufacturing process would help make great strides towards improving the access of such to less developed countries. A recent paper set out to develop a greater understanding of the genetic mechanisms which govern the selection of different antigens of the O1 strain of *V. cholerae* so as to identify mechanisms by which a stable Hikojima strain can be engineered. Doing so would require only one culture of *V. cholerae* bacteria to be produced and inactivated rather than three separate ones while still expressing all 3 O1 serotype antigens. This vaccine was developed by the Holmgren group and induced comparable immunogenicity to Dukoral® in murine studies [307]. Other efforts to simplify the manufacturing process include the engineering of a *V. cholerae* vaccine strain that also expresses CTB in their periplasmic space rather than being

secreted [308]. Another approach to simplifying the expression of rCTB has been to express the antigen in plants, an approach requiring less purification and a more up-scalable, cost effective and durable method for generating rCTB while delivering a product with comparable bioactivity and immunogenicity to that which is produced in engineered bacterial cells [309].

The work that will be conducted in this thesis will hope to provide a logical rationale for the design and development of new and the improvement of existing oral vaccines against enteric diseases. Furthermore, it is hoped that by dissecting the immune mechanisms in the gut which govern the establishment of protective immune responses following oral vaccines

1.19 – Hypothesis

The generation of improved non-living oral vaccines that elicit protective mucosal immune responses requires an integrated approach combining antigens with a suitable delivery technology to avoid gastrointestinal destruction and an effective mucosal adjuvant which engages and enhances early mucosal immune responses.

1.20 – Aims and Objectives

1. To evaluate the ability of the iNKT stimulating ligand α-Galcer to enhance mucosal immune responses following vaccination with a candidate whole cell killed oral vaccine against an enteric pathogen.

2. To determine the benefit to mucosal immune responses of encapsulating whole cell killed oral vaccine formulations in SmPills.

3. To examine the ability of SmPill and α-Galcer to potentiate the protective mucosal immune response against subunit antigens following oral vaccination.

4. To investigate the role of IL-17R in the oral vaccine-mediated protection against oral cholera toxin challenge.

Chapter Two

Materials and Methods

2.1 – Materials

All materials are from Sigma-Aldrich unless otherwise stated.

2.1.1 – General cell culture materials

Complete RPMI

Roswell Park Memorial Institute (RPMI) 1640 medium (Biosera) was supplemented with 2millimolar (mM) L-Glutamine (Gibco), 50units/ml penicillin (Gibco), 50μg/ml streptomycin (Gibco) and 8% (v/v) heat-inactivated (56°C for 30 mins) and filter sterilized fetal calf serum (FCS) (Biosera).

T cell medium

Roswell Park Memorial Institute (RPMI) 1640 medium (Biosera) was supplemented with 0.88 millimolar (mM) L-Glutamine (Gibco), 0.88mM sodium pyruvate (Gibco), 0.04mM β-mercaptoethanol, 0.88% (v/v) MEM non-essential amino acids (Gibco), 0.35% (v/v) 100X MEM Vitamins (Gibco), 4.4units/ml penicillin (Gibco), 4.4μg/ml streptomycin (Gibco) and 10% (v/v) heat-inactivated (56°C for 30 mins) and filter sterilized fetal calf serum (FCS) (Biosera).

0.88% Ammonium chloride (NH_4Cl) red blood lysis solution

8.8g ammonium chloride

1 litre (L) endo-toxin free water (Baxter)

Filter sterilized with 0.22μm syringe-driven filler (Millipore)

2.1.2 – Treatments for Animal Studies and Cell Culture

2.1.2.1 – Immunization/Administration Buffers

0.3M Sodium Bicarbonate Buffer (SBC)

2.52g Sodium Bicarbonate

100ml endotoxin free water (Baxter)

pH adjusted to 9.5 and filter sterilised with 0.22μm syringe-driven filter

pH 5 Buffer

100ml Dulbecco's PBS (Biosera)

pH adjusted to 5.0 and filter sterilised with 0.22μm syringe-driven filter

2.1.2.2 – Adjuvants and Antigens

Cholera Toxin (CT)

Cholera toxin was obtained from Sigma-Aldrich or LIST Biochemicals. Lyophilized CT powder was reconstituted to 10mg/ml (Sigma-Aldrich) or 5mg/ml (LIST Biochemicals) with endo-toxin free water (Baxter).

alpha-galactosylceramide (α-Galcer)

α-Galcer (KRN7000) was obtained from Avanti Lipids. It was solubilized in 100% DMSO (cell culture grade) at 1mg/ml. The suspension was vortexed at high speed for 1min. Next it was heated to 80°C for 2mins. After heating it was sonicated for 5mins in a sonicating water bath after which it was aliquoted and stored at -20°C. Prior to use α-Galcer was thawed and vortexed briefly. Then it was heated to 80°C for 2mins and placed in a sonicating water bath for 10min before being added to a vaccine formulation.

Cholera Toxin B Subunit (CTB)

CTB used for immunisation was obtained from Crucell AG, Sweden.

Formalin killed enterotoxigenic *Escherichia coli* (FK.ETEC) expressing colonization factor antigen I (CFA/I)

FK.ETEC were kindly provided by Prof. Jan Holmgren, University of Göteburg, Sweden. Details regarding the construction of the strain are described here [310].

Formalin killed JS1569 *Vibrio cholerae* expressing Inaba LPS

JS1569 *V. cholerae* expressing Inaba LPS were kindly provided by Prof. Jan Holmgren, University of Göteburg, Sweden. Details on the construction of the strain are describe here [311].

Formalin killed MS1342 (Hikojima) *Vibrio cholerae*

MS1342 *V. cholerae* expressing both Inaba and Ogawa LPS were kindly provided by Prof. Jan Holmgren, University of Göteburg, Sweden. Details on the construction of the strain are described here [307]. Bacteria were used during vaccination and to re-stimulate cells *ex vivo*.

Dukoral®

Dukoral® (Crucell) was obtained through the Trinity Pharmacy, Dublin 2.

2.1.3 – Buffers for the collection of specimens from animal studies

Faecal pellet sample buffer

2.76ml millipore water (mH_20)

2ml 10X PBS (Gibco)

0.4ml 5mg/ml Soybean Trypsin Inhibitor-PBS (STI)

2ml 10% BSA-PBS

1ml 0.5M EDTA (Invitrogen)

200µl 17.5mg/ml PEFAbloc

11.62ml 86% Glycerol

Intestinal wash buffer

3ml 10X PBS

3ml 0.5M EDTA

600µl 5mg/ml STI-PBS

23.4ml mH_20

Perfext sample buffer

200µl 5mg/ml STI-PBS

1ml 0.5M EDTA

200µl 17.5mg/ml PEFAbloc

100µl 10% BSA-PBS

1ml 10X PBS-Tween

7.5 ml mH_2O

2.1.4 – Enzyme-linked immunosorbent assay (ELISA) materials

10X PBS

400g NaCl

58g Na_2HPO_4

10g KH_2PO_4

10g KCl

Made up to a final volume of 5L with mH_2O and brought to pH 7.2

1X PBS

100ml 10X PBS

900ml mH_2O

0.05% PBS-Tween (Wash Buffer)

8995ml mH_2O

1000ml 10X PBS

5ml Tween 20

0.1% BSA-PBS-Tween

1L 0.05% PBS-Tween

1g BSA

Carbonate Buffer

8.4mg $NaHCO_3$, 3.56g Na_2CO_3

Made up to final volume of 5L with mH_2O and brought to pH 9.5.

0.1% BSA

1L mH_2O

1g BSA

1% BSA

1L mH_2O

10g BSA

Phosphate citrate buffer

10.19g $C_6H_8O_7$

14.6g Na_2HPO_4

Made up to a final volume of 1L with mH_2O and brought to pH 5.0

ELISA antibodies (Table 2.1)

Table 2.1 – Antibodies used to measure antigen-specific antibody levels in animal samples

Antibody	Source	Concentration
IgG-Biotin	Sigma-Aldrich	3 µg/ml
IgG1-Biotin	BD Pharmingen	0.1 µg/ml
IgG2a-Biotin	BD Pharmingen	0.1 µg/ml
IgG2b-Biotin	BD Pharmingen	0.1 µg/ml
IgG2c-HRP	AbD Serotec	0.1 µg/ml
IgA-Biotin	BD Pharmingen	0.5 µg/ml

Table 2.2 – Antibodies used to measure cytokine concentrations by ELISA.

Antibody	Source	Concentration	Blocking Solution	Assay Diluent	Top Working Standard	HRP Dilution
IL-17A	Biolegend	Capture - 2µg/ml Detection – 0.4µg/ml	1% BSA	1% BSA	1000pg/ml	1:1000

2.1.5 – SDS PAGE Buffers and Solutions

1.5 M Tris-HCl (pH 8-8.8)

18.15g Tris Base

100ml dH_20 adjusted to pH 8.8

0.5 M Tris-HCl (pH 6.8)

6g Tris Base

100ml dH_20 adjusted to pH 6.8

10% SDS

50g SDS

500ml dH_20

10% Ammonium Persulphate

0.2g Ammonium Persulphate (APS)

2ml dH_20

Sample Buffer

62.5mM Tris pH 6.8

2% w/v SDS

10% Glycerol

0.1% Bromophenol Blue

50mM DDT added moments prior to use

5X Running Buffer

15g Tris Base

72g Glycine

5g SDS

1L dH_20 adjusted to pH 8.3

Table 2.3 – Gel components used in the qualitative analysis of SmPill loading

Component	4% Stacking Gel	15% Resolving Gel
mH_20	12.2ml	10.03ml
30% Bis-Acrylaminde Mix (Biorad)	2.6ml	8.33ml
0.5 M Tris pH 6.8	5ml	---
1.5M Tris pH 8.8	---	6.25ml
10% APS	100µl	150µl
10% SDS	200µl	250µl
TEMED	20µl	12.5µl

Coomassie Blue Stain

1.3g Coomassie Blue

225ml Absolute Alcohol

225ml distilled Water

50ml Acetic Acid

Destaining Solution

225ml Absolute Alcohol

225ml distilled Water

50ml Acetic Acid

2.2 – Methods

2.2.1 – Animals

Female BALB/c and C57BL/6 and male C57BL/6 mice were obtained from Harlan Olac (Bicester, United Kingdom) and were used at 9-16 weeks old. IL-17R KO mice on a C57BL/6 background were obtained from Jackson Laboratories (Maine, United States of America), bred in house and were kindly provided by Dr. Rachel McLoughlin. All animals were housed under specific pathogen free (SPF) conditions Animals were maintained according to the regulations of the European Union and the Irish Medicines Board. Animal studies were approved by the TCD Animal Research Ethics Committee (Ethical Approval Number 091210) and were performed under the appropriate licence (Licence Number B100/3321).

2.2.2 – Cell Culture

Cells were cultured at 37°C with an atmosphere maintained at 95% humidity and 5% CO_2.

2.2.2.1 – Cell viability and counting

The cell suspension was diluted 1:10 (lymph node cells) or 1:50 (splenocytes) with Trypan Blue (Sigma). 10µl of the suspension was loaded onto a KOVA Glasstic cell counter (Hycor Biochemical Inc.), viewed under a light microscope under the x10 objective lens and viability assessed by dye exclusion. The concentration of cells (cells/ml) was then calculated using the formula below.

$$\textit{number of cells/ml} = \textit{cell count} \times \textit{dilution factor } (10 \text{ or } 50) \times 10^4$$

2.2.2.2 – Isolation of Spleen and Lymph Node Cells

Female mice (C57BL/6) were sacrificed by cervical dislocation before removal of spleens and mesenteric lymph nodes. Single cell suspensions were prepared by disrupting tissue through 70μm nylon cells strainers (BD Falcon) with cRPMI 1640 medium. The cells were then centrifuged at 1200 rpm for 5min and cell pellet resuspended in 1ml ammonium chloride (0.08%) for 2min. Cells were then washed in cRPMI 1640 medium and centrifuged again. Cells were then resuspended in 1ml (nodes) or 5ml (spleen) of T cell medium and counted (section 2.2). Splenocytes were plated at $2x10^6$ cells/ml in 200μl T cell medium in 96-well round bottom tissue culture plates (Greiner Bio-One). Lymph node cells were plated at $1x10^6$ cells/ml in 200μl T cell medium in 96-well round bottom tissue culture plates. Both splenocytes and lymph node cells were restimulated *ex vivo* with the appropriate treatments outlined in the relevant experimental sections.

2.2.3 – Enzyme-Linked Immunosorbent Assay (ELISA)

Antigen-specific antibody titres in the various animal specimens and the concentration of cytokines secreted following *ex vivo* re-stimulation of lymphocytes were measured by ELISA.

2.2.3.1 Plate Reading

The absorbance was measured at a wavelength of 492nm using an ELISA plate reader (Thermo Scientific Microplate Reader). The resulting data was analysed using the Scan-IT software (Thermo Scientific). Unknown protein concentrations were determined by reading from a standard curve. Antibody concentrations were expressed as endpoint titres calculated by regression of a curve of OD values versus reciprocal serum levels to a cut-off point of 2 standard deviations.

2.2.3.2 – Cytokine Quantification by ELISA

Splenocytes or lymph node cells were stimulated with either formalin killed MS1432 bacteria, anti-CD3 (BD), or PMA. After incubation for 72 hours, supernatants were transferred from cell culture plates to a fresh 96 well flat-bottomed plates (Greiner Bio-One). All supernatants were stored at -20°C when not in use. Cytokine concentrations in the supernatants were measured by ELISA. Antibody pairs specific for each cytokine were used for immunoassaying. The following cytokines were measured by immunoassay: IL-17.

2.2.3.3 – Standard Cytokine ELISA Protocol

Capture antibodies were made up to the manufacturer's specified dilution (Table 2.2) and a volume of 50μl/well added to high-binding 96 well ELISA plates (Greiner Bio-One). Plates were then incubated overnight at 4°C. After incubation plates were washed in wash buffer (PBS-T) (x4) and tapped dry. Plates were then blocked with the appropriate blocking solution (Table 2.2) for 2 hours at room temperature with gentle shaking. After blocking, plates were

washed in wash buffer (x4) and tapped dry. Cell supernatants were applied to plates at a 1:2 dilution in assay diluent (Table 2.2). A blank triplicate was left on each plate containing the diluent as a blank. Standards were prepared at the starting concentration in the recommended diluent as specified by the manufacturer and transferred to a 96 well plate and serial dilutions (1:2) performed (Table 2.2). All standards and samples were applied to plates at 50µl/well total volume for incubation for 1 hour at room temperature with gentle shaking. After incubation plates were washed with wash buffer (x4) and tapped dry. Detection antibody was then diluted in the diluent as per manufacturer's instructions (Table 2.2) and added to plates at 50µl/well. The plates were left at room temperature for 1 hour with gentle shaking and afterwards washed in wash buffer (x4) and tapped dry. Streptavidin-HRP was diluted according to (Table 2.2) in assay diluent and 50µl/well added to the plate. This was allowed to incubate at room temperature for 30 minutes in the dark with gentle shaking. Plates were once again washed in wash buffer (x4) and once in 1X PBS to remove residual tween and tapped dry. 0.4mg/ml *o*-Phenylenediamine (OPD) substrate was prepared in phosphate citrate buffer (heated to 37°C) containing 4µl H_2O_2 per 10 ml substrate and 50µl added per well. Plates were then stopped by the addition of 25µl/well of 1M H_2SO_4 and read as described in section 2.2.3.1.

2.2.3.4 – Measurement of antigen-specific antibodies

During and after immunisation various animal samples were collected from mice and antibody titres measured by antigen-specific ELISA. Detection antibodies specific for each antigen-specific antibody were used for immunoassaying. The following antibody titres were measured by immunoassay: IgA, IgG, IgG1, IgG2a, IgG2b and IgG2c.

2.2.3.5 – Measurement of CFA/I-specific Antibodies

Titres of CFA/I-specific IgA (BD), IgG, IgG1, IgG2a, IgG2b were determined in faecal pellet, serum, saliva, intestinal wash and perfext samples by using commercially available antibodies (Table 2.1) according to the following method. 50µl per well of 1µg/ml recombinant CFA/I (supplied by Prof. Jan Holmgren) diluted in 1X PBS overnight at 4°C. The ELISA plates were washed three times in 1X PBS and blocked with 200µl 0.1% BSA for 60mins at 37°C. The blocking solution flicked from the plates and these were tapped dry. Samples were diluted in 0.1% BSA-PBS-Tween and added to plates at 50µl per well. The samples were serially diluted across the plate and incubated overnight at 4°C. Plates were washed again 3 times in wash buffer. 50µl per well of detection antibody (Table 2.1) was added to each well and incubated overnight at 4°C. Plates were washed again 3 times in wash buffer and 50µl per well of 700ng/ml HRP-conjugated streptavidin added to plates for 30mins at room temperature in the dark. Plates were washed 4 times in wash buffer and finally once in 1X PBS. 1mg/ml OPD substrate was prepared in phosphate citrate buffer (heated to 37°C) containing 4µl H_2O_2 per 10 ml substrate and 100µl added per well. The ELISA was left to develop at room temperature in the dark. Plates were then stopped by the addition of 25µl/well of 1M H_2SO_4 and read as described in section 2.2.3.1.

2.2.3.6 – Measurement of CTB-specific Antibodies

Titres of CTB-specific IgA, IgG, IgG1, IgG2a, IgG2b, IgG2c were determined in faecal pellet, serum, saliva and perfext samples by using commercially available antibodies (Table 2.1) according to the following method. 96-well medium binding ELISA plates (Greiner Bio-One) were coated with 50μl per well of 0.3 nmol/ml GM1 ganglioside diluted in 1X PBS overnight at 4°C. The ELISA plates were washed once in 1X PBS and blocked with 200μl 0.1% BSA for 30mins at 37°C. The blocking solution was washed from the plates once with 1X PBS. After washing 50μl per well of 0.5μg/ml CTB was added and incubated for 60min at room temperature. The plates were then washed 3 times in wash buffer. Samples were diluted in 0.1% BSA-PBS-Tween and added to plates at 50μl per well. The samples were serially diluted across the plate and incubated overnight at 4°C. Plates were washed again 3 times in wash buffer. 50μl per well of detection antibody (Table 2.1) was added to each well and incubated overnight at 4°C. Plates were washed again 3 times in wash buffer and 50μl per well of 700ng/ml HRP-conjugated streptavidin added to plates for 30mins at room temperature in the dark. Plates were washed 4 times in wash buffer and finally once in 1X PBS. 1mg/ml OPD substrate was prepared in phosphate citrate buffer (heated to 37°C) containing 4μl H_2O_2 per 10 ml substrate and 100μl added per well. The ELISA was left to develop at room temperature in the dark. Plates were then stopped by the addition of 25μl/well of 1M H_2SO_4 and read as described in section 2.2.3.1.

2.2.3.7 – Measurement of LPS-specific Antibodies

Titres of Inaba or Ogawa LPS-specific IgA, IgG, IgG1 were determined in faecal pellet, serum and perfext samples by using commercially available antibodies (Table 2.1) according to the following method. 96-well high binding ELISA plates (Greiner Bio-One) were coated with 50μl per well of 5μg/ml Inaba or Ogawa LPS (supplied by Prof. Jan Holmgren) diluted in 1X PBS overnight at 4°C. The ELISA plates were washed three times in 1X PBS and blocked with 200μl 0.1% BSA for 60mins at 37°C. The blocking solution flicked from the plates and these were tapped dry. Samples were diluted in 0.1% BSA-PBS-Tween and added to plates at 50μl per well. The samples were serially diluted across the plate and incubated overnight at 4°C. Plates were washed again 3 times in wash buffer. 50μl per well of detection antibody (Table 2.1) was added to each well and incubated overnight at 4°C. Plates were washed again 3 times in wash buffer and 50μl per well of 700ng/ml HRP-conjugated streptavidin added to plates for 30mins at room temperature in the dark. Plates were washed 4 times in wash buffer and finally once in 1X PBS. 1mg/ml OPD substrate was prepared in phosphate citrate buffer (heated to 37°C) containing 4μl H_2O_2 per 10 ml substrate and 100μl added per well. The ELISA was left to develop at room temperature in the dark. Plates were then stopped by the addition of 25μl/well of 1M H_2SO_4 and read as described in section 2.2.3.1.

2.2.4 – SDS Page and Coomassie Blue Staining

2.2.4.1 – Extraction of Antigens from SmPills

SmPills corresponding to the doses indicated in the corresponding (Figure legends were placed in Dulbecco's PBS and heated to 50°C for 60mins to dissolve the beads. After dissolving the samples were vortexed and centrifuged at maximum g for 5mins. Supernatants were transferred into fresh tubes and mixed with de-naturing sample buffer. Samples were boiled for 3 mins at 100°C.

2.2.4.2 – SDS-PAGE

The stop buffer was poured and allowed to set for 30 minutes before the resolving and stacking gels were poured respectively (Table 2.3). Once the resolving gel was set, 20μl of sample was loaded into each well in addition to 8μl of a protein molecular weight ladder (Biorad Dual Colour 5-75kDa). Once the samples were loaded, the gel was run at 120 volts (V) for 90 minutes in running buffer.

2.2.4.3 – Coomassie Blue Staining

After running SDS-PAGE gels were washed in mH_2O for 5 mins prior to staining with Coomassie Blue overnight and room temperature with light rocking. The following day gels were washed with de-staining solution several times until the protein bands appeared vibrant without any background interference. The gel was scanned on an Osiris gel scanner. CTB was located at ~12kDa and quantities present in SmPills compared to prepared standards of known concentration based on the visual intensity of the bands.

2.2.5 – Animal Studies

2.2.5.1 – Oral administration of solutions

Food was removed 1 hour prior to immunisation. Mice were immunised by oral gavage with 38.1mm 18 g stainless steel curved feeding needles (Harvard Apparatus) with 200μl of 0.3M Sodium Bicarbonate solution at pH 9.5 containing antigens and adjuvants at the concentrations stated in the corresponding figure legends. Food was returned 30 min later.

For the two MS1346 studies in chapter 4 an amended oral immunisation protocol was used. Briefly mice were administered 200μl of 0.3M Sodium Bicarbonate solution at pH 9.5. 20 mins later antigens and adjuvants were administered in 200μl 1XPBS as stated in the corresponding figure legends. Food was returned 30 min later.

2.2.5.2 – Oral administration of SmPills

Food was removed 1 hour prior to immunisation. Mice were first anesthetized with inhalation iso-flurane (VetTech). Mice were immunised with 2-3 SmPills (depending on doses of antigens and adjuvants combinations obtained during SmPill manufacture was indicated in

corresponding figure legends). Briefly the particles were loaded into the silicon tip of a 17 gauge flexible Teflon feeding tube (Agnthos) and gentle fed down the oesophagus of the anesthetized mouse. The particle was released by gently pushing down the plunger of a 1ml syringe (Braun Injekt-F) containing 50µl of pH 5 buffer. After immunisation mice were monitored to ensure recovery from anaesthesia and food was then returned 30 min later.

2.2.5.3 – Collection of Faecal Pellets

Mice were placed into individual cages and 5 fresh faecal pellets collected and placed into a tube containing 500µl of cold faecal pellet buffer and samples kept on ice for 2 hours. Samples were then emulsified with a yellow inoculation needle (Greiner Bio-one). Samples were then centrifuged at 15,400g for 5min at 4°C. Supernatants were transferred to fresh eppendorf tubes and stored at -80°C until further use.

2.2.5.4 – Tail bleeding and serum collection

Blood was obtained from mice by incision into the tail vein and collection in eppendorf tubes. Samples were left to coagulate overnight at 4°C and then centrifuged at 9,200g for 10min at room temperature. The serum was removed into a fresh eppendorf and stored at -80°C until further use.

2.2.5.5 – Collection of saliva

Mice were injected intraperitoneally with 0.1mg of Pilocarpine-HCl (TOCRIS bioscience) in 100µl Dulbecco's PBS and carefully observed for the onset of salivation. Saliva was collected in 7ml tubes for the duration of salivation and placed on ice after. 7ml tubes were placed into 50ml conical tubes and centrifuged for 5mins at 1,500g at 4°C. Saliva was transferred to fresh eppendorf tubes and stored at -20°C until further use.

2.2.5.6 – Intestinal washes

Isolated intestines where flushed through with 6ml of intestinal wash buffer for small intestinal washes or 1ml for large intestinal washes respectively. Intestinal washes were stored on ice until further processing. Samples were centrifuged at 1,500g for 10min at 4°C and 2ml of small intestinal and 0.8ml of large intestinal supernatant were transferred to fresh eppendorf tubes and 100mM phenylmethanesulfonylfluoride (PMSF) added to all samples with all work being carried out on ice. Samples were again centrifuged at 18,000g for 10 min at 4°C before being transferred to fresh eppendorf tubes containing a further 100mM PMSF. 50µl of FCS and 5µl of 1% sodium azide solution were finally added per 1ml of intestinal supernatant on ice. Samples were stored at -20°C until further use.

2.2.5.7 – Perfusion-extraction (Perfext)

Fresh Perfext sample buffer (270µl per tissue sample) was prepared on the day of the experiment, added to eppendorf tubes, the tubes weighed and placed on ice. Mice were sacrificed by cervical dislocation and the rib cage removed. The mouse was perfused with 20ml 0.1% Heparin PBS using a 23G needle (BD) and 20ml syringe (BD) with 15ml though

the heart and the remaining 5ml through the caudal mesenteric arteries. Following perfusion the excess perfusate was removed with a paper towel and the intestine removed from the junction at the base of the stomach and from the anus. The GIT was trimmed of fat and mesentery and the duodenum removed. The intestine was separated into the desired segment length and opened longitudally. Faeces were scraped out and the intestine was washed in ice cold Dulbecco's PBS and dried on a fresh paper towel prior to placing it into an eppendorf containing perfext sample buffer and stored on ice. The tubes were weighed again and 30μl of 20% Saponin-PBS was added into each tube and vortexed. The tubes were incubated overnight at 4°C. The next day tubes were centrifuged at maximum g for 10min and the supernatant removed and added into a fresh eppendorf and stored at -20°C until use.

2.2.6 – Oral cholera toxin challenge model; Challenge and readouts

Food was removed from mice for 12 hours prior to CT challenge. Mice were given 100μl of 0.3M sodium bicarbonate solution at pH 9.5 by oral gavage. After 20 min mice were given 200μl of 20μg CT in 0.3M sodium bicarbonate solution at pH 9.5. Balb/c mice were left without water and food for 16 hours after CT challenge prior to euthanasia. C57BL/6 mice were left without food but with water *ad libitum* for 6 hours after CT challenge prior to euthanasia.

2.2.6.1 – Dissection of GIT

Mice were sacrificed by cervical dislocation. The peritoneal and abdominal cavity was opened longitudally and the small intestines cut at the junction between the stomach and duodenum. Carefully the intestine was pulled out of the abdominal cavity while also removing the surrounding fat and mesenteric tissue. Care was taken not to rip the intestine or puncture them as this could cause leakage of fluids and alter weight ratios later.

2.2.6.2 – Intestinal Photographs

Intestines were removed from petri dishes and dabbed dry with fresh tissue. The intestines were next placed on a clean, white surface and several photographs taken without flash and on the macro setting.

2.2.6.3 – Separation of intestinal components and weighing of caeca

First a petri dish was weighed with the lid and the weight was noted. While still in the petri dish the small intestine and large intestine was separated from the caecum, to avoid loss of caecum contents, and the wet weight recorded. The caeca were dried for 7 days in an oven at 37°C. After drying the whole petri dish was weighed again to determine the dry weight of the caeca.

2.2.6.4 – Percentage fluid in caecum

In order to determine the percentage water lost during the drying process of the caeca the following formula was employed.

$$Percentage\ Fluid\ in\ Cecum = \frac{(dry\ weight - dish\ weight)}{(wet\ weight - dish\ weight)}\ x\ 100$$

2.2.6.5 – Determining the presence of CT toxicity in mouse intestines

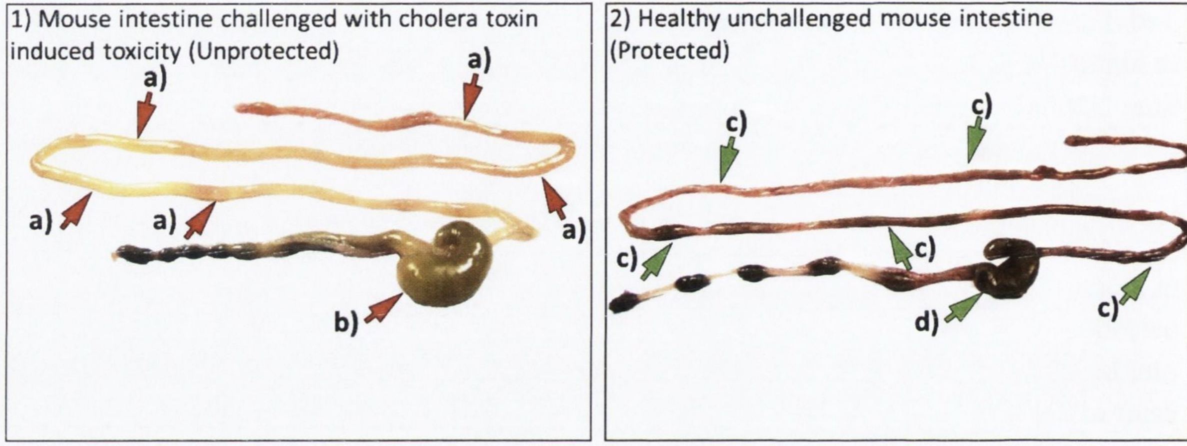


Figure 2.1 – A comparison of cholera toxin induced fluid accumulation in a challenged mouse intestine versus a healthy mouse intestine. 1) Image represents the intestine of a female C57BL/6 mouse 6 hours after oral administration of 20μg CT. a) shows fluid accumulation in the small intestine and absence of dark patches of solid faecal matter. b) illustrates the profuse fluid accumulation and lack of solid stool in the caecum. In both a) and b) the intestine appears in a yellow/greenish hue. 2) This image represents the normal anatomy of a health C57BL/6 intestine. c) a red coloured and thin small intestine with patches of solid faecal matter is observed in healthy mice. d) the caecum is contracted and contains solid faecal matter. Intestine 1 is deemed to be susceptible to oral CT mediated toxicity (unprotected) while intestine 2 is considered healthy and if the mouse was challenged with CT protected.

2.2.6.6 – Scoring of Intestine photographs

Intestinal photographs were randomized and sent to 4 assessors to determine susceptibility to CT-mediated toxicity. Presence or absence of fluid accumulation in the small intestine and caecum was used as a measure. Results are expressed as Percentage of mice protected which correlates to the number of mice which were deemed to have an absence of fluid accumulation using the above described guidelines. Results were calculated using the following example.

4 assessors reviewed photographs of three intestines. For intestine A and B 4/4 assessors agreed to the absence of fluid. However 1 assessor indicated to the presence of fluid in intestine C. Therefore in the case of intestine A and B, 4/4 (i.e. 100%) agree protected against oral cholera challenge while only 3/4 (i.e. 75%) of assessors agree there is an absence of fluid in intestine C. Total results are thus calculated by adding up the percentage of assessors agreeing to the absence of fluid accumulation for all intestinal photographs in the group and dividing this by the total number of mice in the group as follows:

$$\frac{(100\% + 100\% + 75\%)}{3} = 91.7\% \text{ Total protection}$$

2.2.7 – Statistical analysis

Statistical analysis was performed using Graphpad Prism 5 software. The means for three or more groups were compared by one-way ANOVA. Where significant differences were found, the Tukey-Kramer multiple comparisons test was used to identify differences between individual groups. The means for two groups were compared using an unpaired Student's t test. For graphs representing changes in antibody titres over time the area under the curve (AUC) for each individual mouse was determined. These were then represented as the mean value for the AUC of the group and the appropriate statistical analysis performed to identify differences between individual groups as outlined above.

Chapter Three

Evaluation of an integrated approach to oral vaccination

3.1 – Introduction

3.1.1 – Oral Vaccines, the good, the bad, the ugly.

The merits of oral vaccines are evident when one considers the success of oral polio vaccine (OPV). The lower cost per dose and ease of administration of OPV compared to the injectable inactivated polio vaccine (IPV) has demonstrated the advantages of the oral route over the injectable route especially for the induction of mucosal effector immune responses. However, in rare cases the attenuated virus in OPV can revert to a virulent strain and cause vaccine-associated paralytic poliomyelitis (VAPP) [3]. This potential danger associated with attenuated oral vaccines has led to the preferable development of either whole cell killed (WCK) or subunit oral vaccine antigens [12, 312]. While attenuated vaccines often establish a weak, local infection which adequately serves to stimulate the immune response, subunit antigens require either the addition of powerful adjuvants to stimulate the immune system or high doses of antigen [12, 312]. Another challenge facing oral vaccination is the possible destruction of vaccine components in the harsh acidic environment of the stomach or enzymatic degradation in the intestine [12, 312]. Furthermore, it is also beneficial to target and often control the release of oral vaccines in an attempt to more accurately imitate the kinetics of a live infection [15]. Therefore, in order to improve the efficacy of oral vaccines these challenges must be addressed.

3.1.2 - The Single Multiple Pill®, a novel oral delivery system

The Single-Multiple Pill® (SmPill) is an oral drug delivery system developed by Sigmoid Pharma Ltd (Figure 3.1.1). The rationale behind the development of this technology was to create a delivery system capable of transporting pharmaceutical agents to specific areas of the gastrointestinal tract (GIT). Firstly, the system would prevent damage to the payload by acids in the stomach and enzymatic activity in the duodenum, secondly, it enables the targeted

delivery of contents to discrete regions of the GIT and thirdly it would incorporate an intrinsic release mechanism that would enhance both uptake and distribution in the target area. The structure of the SmPill is the key to understanding how this technology can perform its function (Figure 3.1.2). The outside of the SmPill is surrounded by an enteric polymer coating which remains stable at the low gastric pH (Figure 3.1.2 a). By varying the composition of this polymer it is possible to determine the length of time which it takes to degrade the enteric coat, thus allowing the SmPill's release location to be controlled (Figure 3.1.4). Finally, upon exposure to the higher pH of the more distal portions of the small intestine this polymer begins to degrade, exposing the gelatin core (Figure 3.1.2 b). This gelatin core is composed of a porous sponge-shaped structure that contains many droplets of a drug or vaccine and a solubilizing agent which together form an emulsion (Figure 3.1.1 b & 3.1.2 b & c). The solubilizing agent currently used in the manufacture of the SmPill is Solutol (Figure 3.1.3) which has previously been shown to aid in the bioavailability of poorly soluble drugs [313]. However, the nature of the solubilisation agent can be varied, depending on the requirements of the payload, thus making it a very versatile delivery system. Solutol was chosen during the initial formulation stages as it is well characterised and commercially available [313]. When the gelatin core is degraded by the natural action of the digestive system, it is postulated to release a multitude of droplets of the emulsion, which are then taken up by the intestinal tissue (Figure 3.1.4). The primary application of this technology was foreseen to be the delivery of the anti-inflammatory drug cyclosporine to inflamed regions of the intestine in patients with inflammatory bowel diseases. However, it was precisely these attributes of payload protection, GIT targeting and enhanced delivery that led to the investigation of the SmPill as a possible oral vaccine delivery system. Preliminary studies concluded that the SmPill indeed was a viable candidate for mucosal vaccine delivery (Figure 3.1.7 b).

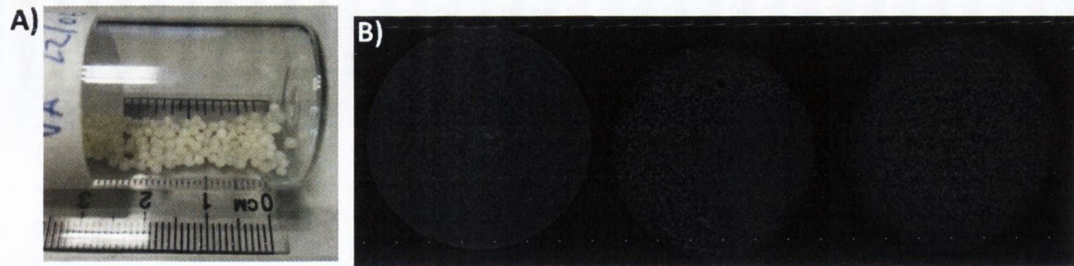


Figure 3.1.1 – The SmPill; a novel oral delivery system. SmPills are approximately 1mm in diameter and are composed of a gelatin core which has a honeycomb structure. These pores contain the payload of the SmPill which is solubilized in a surfactant emulsion. (*Images courtesy of Monica Rosa, Sigmoid Pharma Ltd.*)

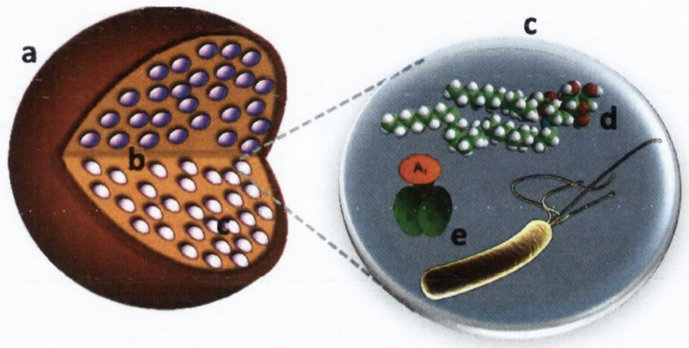


Figure 3.1.2 – The structure of the SmPill. The SmPill is composed of an external enteric coat (a) which surrounds a gelatin core (b). Trapped within this gelatin core are a large number of droplets (c) composed of a surfactant which contains the payload in an emulsion (d & e).

Figure 3.1.3 – Chemical structure of Solutol HS 15 (polyethyleneglycol 660 12-hydroxystearate). The Solutol molecule contains both hydrophobic and hydrophilic moieties allowing it to solubilise hydrophobic compounds in an aqueous medium. Solutol is thought to form micelle-like structures in an aqueous solution which may explain the mechanism behind its solubilizing properties [313].

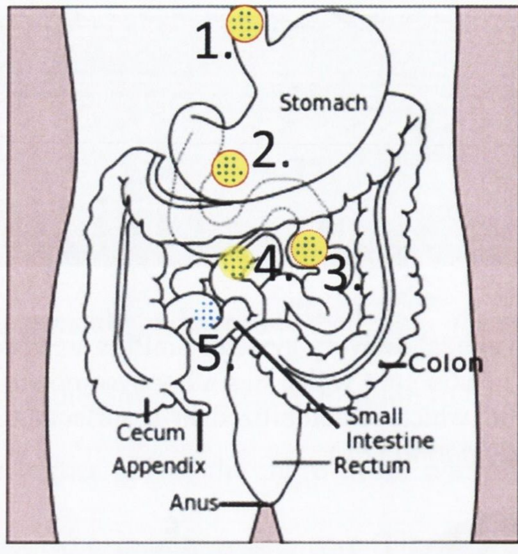


Figure 3.1.4 – SmPills facilitates the transit of encapsulated payloads through the stomach and duodenum by protecting the cargo from acidic and enzymatic degradation. The image above outlines the hypothesised mechanisms of payload protection and delivery by SmPills in the intestine.

3.1.3 – Construction of CFA/I overexpressing non-toxic *E.coli* strain as a strategy to manufacture a more immunogenic whole cell killed ETEC vaccine.

Enterotoxogenic *E.coli* (ETEC) is an enteric species of pathogenic *E.coli* [206, 207]. The global distribution of regions in which ETEC remains endemic is staggering (Figure 1.24). ETEC causes a profuse watery diarrhoea and are spread by contaminated drinking water and food [226, 227], which results in rapid dehydration and prostration within hours [254]. Children are by far the biggest risk group for ETEC infection with 300,000-500,000 cases in those under five years of age reported in the developing world annually [233]. Aside from causing disease in residents of endemic regions ETEC is the leading cause of Traveller's diarrhoea, which often affects non-resident travellers, military personnel and foreign aid workers travelling to endemic regions (Figure 1.25) [228]. Furthermore, humans are not the only hosts of ETEC, with animals of agricultural significance also susceptible to the disease

[237]. To date there is no licenced and effective oral ETEC vaccine (OEV). However, this is not for lack of trying (section 1.15).

Many studies have suggested that mucosal antibodies targeted against fimbriae can prevent colonization of the GIT by the bacterium and thus protect from disease [257]. Human studies involving the oral vaccination of individuals with an OEV composed of formalin killed-strains of ETEC expressing combinations of colonization factor antigen (CFA)/I, II and IV together with purified cholera toxin B subunit (CTB) found the vaccine to be safe and immunogenic in travellers whilst protecting them from diarrhoea [259]. In contrast there was no significant protection elicited in children and infants given this vaccine in an ETEC endemic area [257]. Furthermore, this vaccine induced several incidents of vomiting in these subjects. These results highlighted the need for a safer and more immunogenic oral ETEC vaccine which could protect residents in an endemic region [259, 265].

As discussed in section 1.15.3, CFA/I was identified as an antigen of interest as formalin inactivated strains expressing CFA/I induced strong intestinal immunity to homologous ETEC strains. To enhance immunogenicity in the GIT, Tobias *et al* constructed a novel strain of *E.coli* overexpressing the antigenic ETEC CFA/I colonization factor, which has been shown to be highly conserved across many strains of ETEC in various endemic areas including South America, Africa and Asia (Figure 3.1.6) [314, 315].

It was hoped that overexpressing CFA/I on non-toxigenic strains of *E.coli* could help increase safety and enhance immunogenicity. Tobias *et al* clearly demonstrated the enhanced immunogenicity of these bacteria versus a CFA/I positive reference ETEC strain (Figure 3.1.5) [310]. Furthermore, the use of a non-toxic *E.coli* strain to serve as a stable platform for expressing the CFA/I antigen acts to enhance the safety profile of this vaccine. These encouraging data made this formalin-killed CFA/I overexpressing whole cell *E.coli*

(FK.ETEC) a prime candidate to evaluate the capacity of SmPills to function as an oral vaccine delivery system.

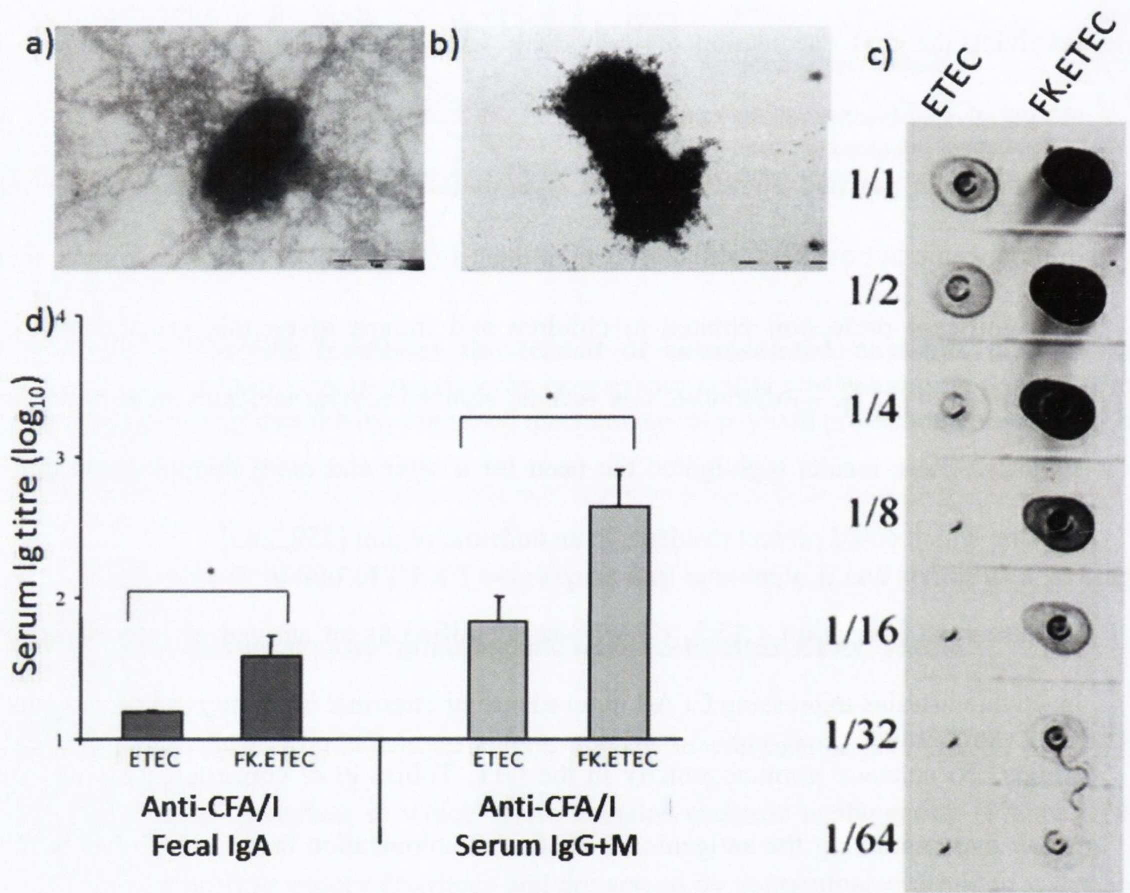


Figure 3.1.5 – Construction and evaluation of a novel CFA/I over-expressing non-toxic *E.coli* strain, a candidate antigen for an oral ETEC vaccine. Electron micrographs of anti-CFA/I Mab immunogold-labelled bacterial cells of the FK.ETEC (a) and the ETEC reference strain (b). (c) A comparison of CFA/I expression using the dot blot assay between an ETEC reference strain and FK.ETEC. (d) IgA, IgG and IgM anti-CFA/I serum titres were measured after mice were orally vaccinated with either ETEC reference strain or FK.ETEC in solution together with 10μg CT [310].

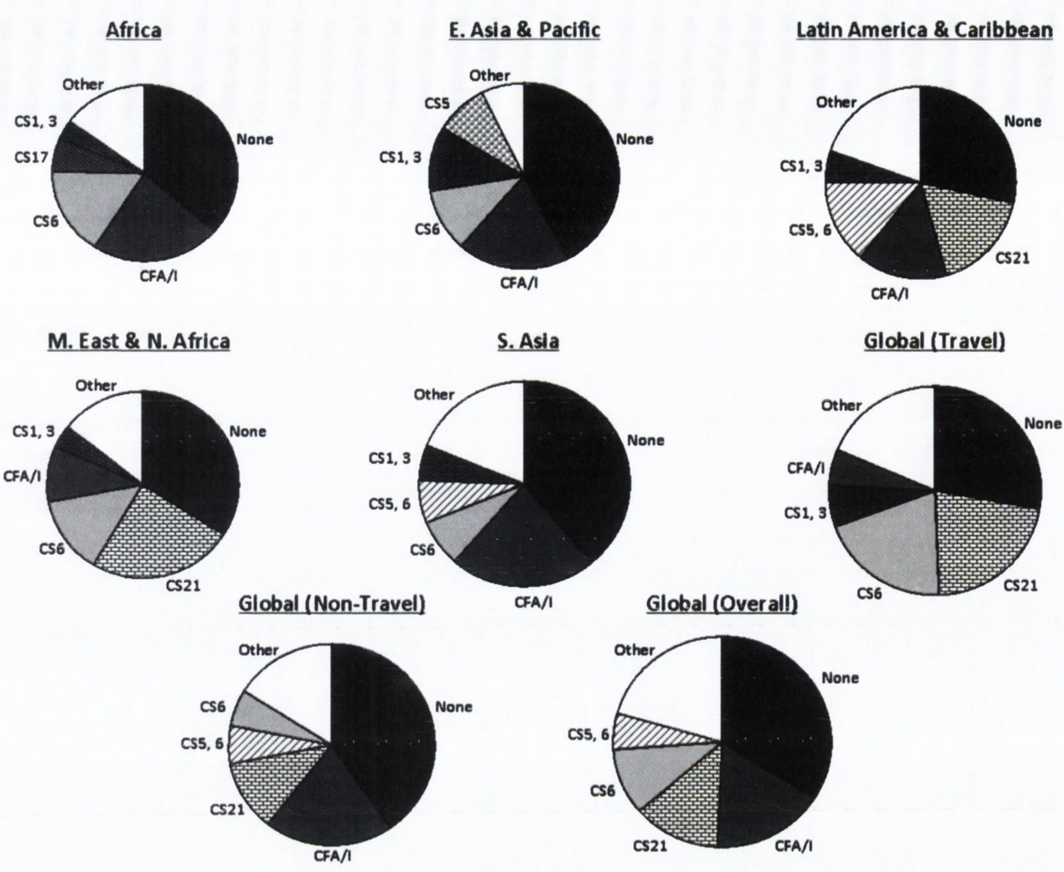


Figure 3.1.6 – ETEC colonization factor prevalence by region and travel/non-travel population. Regional estimates describe ETEC colonization factor distribution in all included studies. Global estimates describe estimates obtained from all included studies (i.e., overall), and are also stratified by travel and non-travel populations [314].

3.1.4 – The synthetic lipid antigen α-Galcer is a potent adjuvant that boosts mucosal immunity after oral vaccination.

In order to elicit a local intestinal immune response several obstacles need to be addressed. Firstly, one must overcome the inherent predisposition towards tolerance in the gut and secondly, address the often weak immunogenicity of antigens delivered by the oral route. Therefore it is necessary to incorporate a mucosal adjuvant into the vaccine formulation in order to overcome these challenges [12]. The most potent mucosal adjuvants are those based on native or modified versions of the heat-labile toxins CT and LT [138]. However, many of these adjuvants have been shown to be either too toxic for use in humans, or previous results

from mucosal vaccination trials have led to viewing these in a negative light by regulatory authorities (Section 1.11.1). Therefore there is a pressing need to identify other potent and safe orally active adjuvants. TLR agonists are regarded as highly effective injectable adjuvants; however, these failed to elicit comparable immune stimulation to CT, the gold standard experimental mucosal adjuvant, when delivered orally (Figure 3.1.7 a).

Recently, interest in the immune-modulating potential of NKT cells has led to studies evaluating the capacity of NKT cell-activating ligands to act as novel adjuvants [188]. A subset of NKT cells known as invariant natural killer (iNKT) cells are categorized by their expression of an invariant T cell receptor and which upon stimulation produce many T cell polarising cytokines (section 1.12.2) [174]. CD1d (which is expressed on several types of intestinal APCs, B cells and IECs [190]) presents antigens to cells expressing the invariant T cell receptor and unlike MHC class one and two molecules, CD1d restricted antigens are not peptides but lipids (Section 1.12.1) [316]. Alpha-Galactosylceramide (α-Galcer) is a sphingolipid isolated from the marine sponge species *Agelas mauritianus* and is most commonly available in the form of KRN7000 which comprises an α-linked galactose head group and a ceramide base composed of a 28-carbon phytosphingosine chain and a 26-carbon acyl chain (Figure 1.10), and was initially investigated for its anti-tumour properties [196]. α-Galcer has been shown to bind to both murine and human CD1d [179]. The immune potentiating effects of α-Galcer have been documented in several studies after both systemic and mucosal administration [188]. During initial screening studies α-Galcer exhibited strong adjuvant properties in SmPills comparable to CT in solution (Figure 3.1.7 b). α-Galcer also elicited stronger antibody titres than the adjuvants monophosphoryl lipid A (MPLA) and QuilA (Figure 3.1.7 b). We also showed that α-Galcer was well tolerated orally in mice and was shown to exhibit no symptoms of diarrhoea in contrast to CT (Figure 3.1.8). With these properties in mind α-Galcer was chosen as a suitable candidate for an oral vaccine adjuvant.

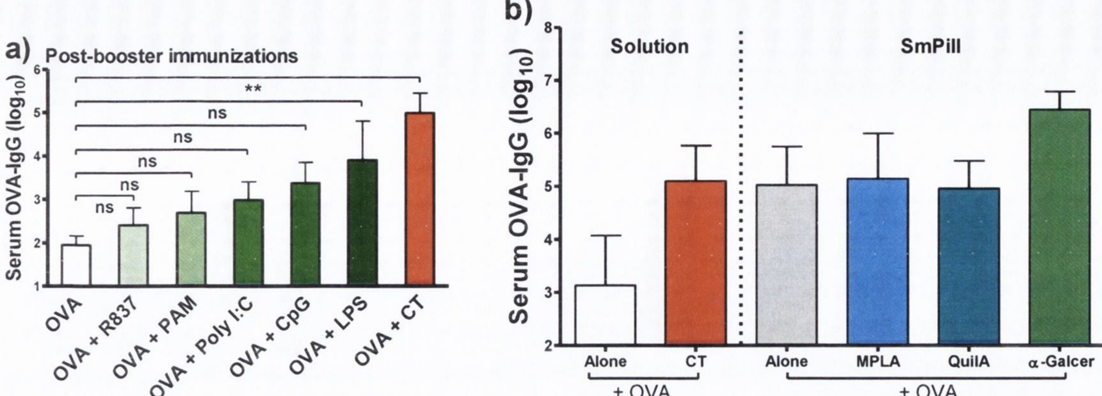


Figure 3.1.7 – TLR agonists are poor inducers of humoral immunity after oral vaccination while α-Galcer effectively drives antibody production. (a) TLR agonists (50μg) or CT (10μg) were orally co-administered in solution with ovalbumin (OVA) (100μg) on 3 consecutive days and boosted four weeks later for a further 3 consecutive days. 7 days following the final boost serum samples were obtained and IgG were determined. (b) Mice were orally vaccinated with OVA (200μg) alone or together with CT (10μg) or with SmPills containing OVA (200μg) alone or with either α-Galcer (10μg), monophosphoryl lipid A (MPLA) (10μg) or QuilA (10μg) for 2 consecutive days 3 weeks apart. On day 14, mice were intra-peritoneally boosted with alum and OVA. Serum was obtained a week later and OVA-specific IgG titres determined. Oral vaccination with SmPills containing OVA alone or co-administered with MPLA, α-Galcer or QuilA elicited enhanced serum IgG titres compared to OVA alone in solution. However only α-Galcer elicited stronger titres than OVA co-administered with CT in solution. (Experiment performed by Dr. Edel McNeela, *unpublished data*).

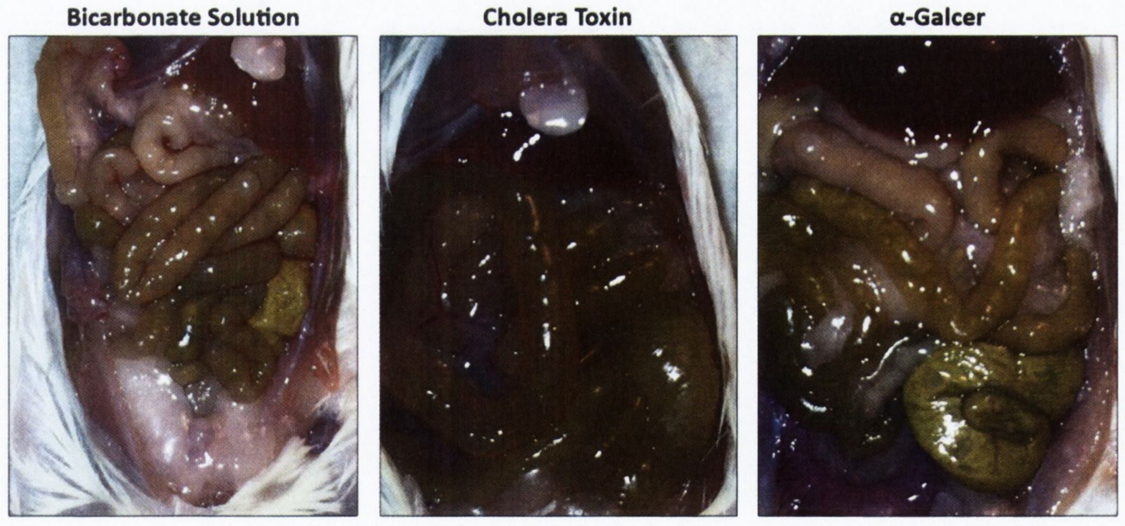


Figure 3.1.8 – α-Galcer does not induce diarrhoea after oral administration. BALB/c mice were orally administered sodium bicarbonate alone or together with either 10μg CT or 10μg α-Galcer in solution. 6 hours post administration mice were sacrificed and the abdominal cavity opened. Photographs of the abdominal cavity were taken. (Experiment performed together with Craig McEntee, *unpublished data*).

3.2 – Hypothesis, Aims and Objectives

- Hypothesis -

Co-formulation of a candidate antigen together with a potent mucosal adjuvant in a suitable delivery vehicle can enhance the efficacy oral vaccine efficacy.

- Aims and Objectives -

1. To determine the potential of orally administered FK.ETEC and α-Galcer to induce mucosal and systemic antibody responses against CFA/I.

2. To compare the relative efficacy of the oral delivery of FK.ETEC and α-Galcer in either solution or encapsulated in SmPills.

3. To perform a comprehensive investigation into the induction of mucosal and serum antibody responses elicited by orally delivered FK.ETEC alone or with α-Galcer in SmPills.

3.3 – Results

3.3.1 – Comparable antigen-specific faecal antibody titres are elicited after oral vaccination with FK.ETEC adjuvanted with either CT or α-Galcer.

ETEC infection occurs in the GIT and IgA antibodies against colonisation factors (CFs) can prevent adhesion, infection and aid clearance of the pathogen by mucus secretions [257]. IgA antibodies present in faecal pellets are used as a representative measure of the local IgA responses in the lumen of the GIT [317].

In order to assess the mucosal adjuvanticity of α-Galcer and compare it to that of CT, a well-documented mucosal adjuvant [134], mice were vaccinated as per figure 3.5.1 with sodium bicarbonate (SBC) solution alone or in combination with $3x10^8$ FK.ETEC with or without 10μg α-Galcer or 10μg CT per mouse. Faecal pellets were collected on days 13, 27 and 34 and CFA/I-specific IgA (Figure 3.5.2) and IgG (Figure 3.5.3) titres determined by end-point ELISA.

Analysis of faecal pellets on days 13, 27 and 34 showed that FK.ETEC adjuvanted with either CT or α-Galcer induced comparable CFA/I-specific IgA titres that were higher than FK.ETEC bacteria alone after 3 rounds of vaccination (Figure 3.5.2 a, b and c). However, only the addition of α-Galcer resulted in a significant increase in titres compared to FK.ETEC alone after three rounds of vaccination (Figure 3.5.2 c). The kinetics of CFA/I-specific antibody induction in both CT and α-Galcer groups followed a similar pattern over the course of the experiment (Figure 3.5.2 d). No significant differences in the area under the curve (AUC) were found between CT and α-Galcer after 3 rounds of oral vaccinations (Figure 3.5.2 d).

Measurement of CFA/I specific IgG titres in faecal pellets showed that the inclusion of either CT or α-Galcer as adjuvants with FK.ETEC induced significantly stronger CFA/I-specific

IgG titres than FK.ETEC alone after three rounds of vaccination (Figure 3.5.3 c). No significant differences were found between the ability of α-Galcer or CT to enhance faecal IgG titres after 3 rounds of vaccination (Figure 3.5.3 c). Both adjuvants displayed similar induction profiles of CFA/I-specific IgG antibodies after one, two and three rounds of vaccination and no significant differences in were found in AUC analysis (Figure 3.5.3 d).

3.3.2 – Oral vaccination with FK.ETEC and α-Galcer or CT promotes comparable antigen-specific antibody secretion into the small intestinal lumen.

ETEC is an enteric pathogen which primarily infects the small intestine. Therefore a strong local antibody response at this location could help prevent colonization and aid clearance of the pathogen. Vaccination with FK.ETEC and α-Galcer was shown to elicit comparable faecal antibody titres to FK.ETEC and CT in mice (Figure 3.5.2 & 3.5.3).

To investigate the ability of the FK.ETEC and α-Galcer formulation to induce local intestinal secretory antibody responses, mice were orally vaccinated as in 3.3.1 and 35 days after the initial vaccination mice were given three oral doses of polyethylene glycol (PEG) to induce mucosal secretions. Mice were then sacrificed and intestinal washes performed on the small intestine and CFA/I-specific IgA (Figure 3.5.4 a) and IgG1 (Figure 3.5.4 b) end-point ELISAs performed on this wash.

FK.ETEC solutions adjuvanted with α-Galcer induced marginally higher CFA/I-specific IgA titres when compared to FK.ETEC alone (Figure 3.5.4 a). However, this difference was not significant. CFA/I-specific IgG1 titres were not significantly elevated by coadministration of CT or α-Galcer compared to FK.ETEC alone (Figure 3.5.4 b).

3.3.3 – The co-administration of either α-Galcer or CT with FK.ETEC did not increase CFA/I-specific serum IgG titres after oral vaccination.

The ability of an oral vaccine to elicit systemic IgG responses is often taken as an indicator of efficacy in human trials.

To investigate the ability of oral FK.ETEC vaccine formulations to induce serum IgG responses, mice were vaccinated as in 3.3.1. Serum was obtained on days 13, 27 and 35 and CFA/I-specific IgG responses were measured by end-point ELISA (Figure 3.5.5).

Co-administration of CT or α-Galcer did not lead to a significant increase in CFA/I-specific IgG titres at any of the three time points (Figure 3.5.5 a, b and c). Similarly, no differences were found when AUC was analysed (Figure 3.5.5 d).

3.3.4 – Oral delivery of FK.ETEC and α-Galcer in SmPills enhances faecal antibody titres over administration in solution.

Oral vaccination with FK.ETEC and α-Galcer in solution elicited strong antigen-specific faecal pellet antibody responses (Figure 3.5.2 and 3.5.3). The efficacy of some oral vaccines can be improved when the vaccine components are protected during their transit through the harsh gastric environment of the stomach and the enzymatically active region of the duodenum by utilising a suitable delivery system [312].

To examine if there is a benefit to encapsulating the FK.ETEC and α-Galcer formulation in SmPills, mice were vaccinated as per figure 3.5.1 with SBC solution alone or in combination with $3x10^8$ FK.ETEC with 10μg α-Galcer in solution. SmPills containing $3x10^8$ FK.ETEC with 10μg α-Galcer were prepared and delivered orally to mice. On days 13, 27 and 34 faecal pellets were collected and end-point ELISA performed for CFA/I-specific IgA (Figure 3.5.6) and IgG (Figure 3.5.7).

Analysis of faecal pellets collected on day 34 showed a significant increase in CFA/I-specific IgA in mice vaccinated with SmPill formulations versus those in the solution group (Figure 3.5.6 c). Observation of the kinetics of the CFA/I-specific IgA response indicated a stronger induction and sustained response in mice vaccinated with SmPill versus identical formulations in solution indicated by a significantly higher AUC (Figure 3.5.6 d).

Analysis of end-point titres at individual time points did not reveal significant differences in CFA/I-specific IgG titres between mice vaccinated with FK.ETEC and α-Galcer in SmPills or in solution on day 35 (Figure 3.5.7 c). However, a significantly higher AUC was determined in mice receiving FK.ETEC and α-Galcer in SmPills over the course of the experiment compared to those vaccinated with FK.ETEC and α-Galcer in solution (Figure 3.5.7 d).

3.3.5 – SmPill formulations of FK.ETEC and α-Galcer elicit marginally stronger small intestinal IgA titres compared to identical formulations in solution.

Mice vaccinated with FK.ETEC and α-Galcer did not display significantly enhanced intestinal wash CFA/I-specific IgA (Figure 3.5.4). However, faecal pellet IgA and IgG was significantly elevated compared to mice vaccinated with FK.ETEC alone (Figure 3.5.2). As IgA secretion into the lumen of the intestine is dependent on the presence of IgA^{+} ASCs in the lamina propria it was determined that the CFA/I-specific IgA content of the intestinal tissue should be examined as this might present a more accurate representation of local intestinal antibody responses than intestinal washes. The perfusion-extraction (Perfext) method is a method commonly employed to evaluate local tissue antibody and cytokine responses [318].

In order to evaluate the benefit of encapsulating vaccine formulations in SmPills to local CFA/I-specific antibody responses in the LP, the site of IgA production, mice were vaccinated as per 3.3.4. 35 days after the initial vaccinations mice were sacrificed and perfused with heparin PBS to exclude serum antibodies and measure local tissue responses. The small and

large intestines were dissected out and a 3cm segment removed from each respectively. Segments were treated with saponin to disrupt the cell membrane and release Ig molecules into the supernatant and end-point ELISAs were performed for CFA/I-specific IgA (Figure 3.5.8) and IgG (Figure 3.5.9).

Vaccination with FK.ETEC and α-Galcer in SmPill led to a small yet not significant increase in antigen-specific IgA titres in the small intestine compared to delivery in solution (Figure 3.5.8 a). CFA/I-specific IgA titres in the large intestinal segments were not elevated in mice vaccinated with SmPills compared to solution (Figure 3.5.8 b).

No enhancement of CFA/I -specific IgG titres in the small intestine (Figure 3.5.9 a) or large intestine (Figure 3.5.9 b) were detected when FK.ETEC and α-Galcer was delivered in SmPills compared to those in solution.

3.3.6 – Delivery of FK.ETEC and α-Galcer in SmPills enhances CFA/I-specific serum IgG titres compared to delivery in solution.

Although ETEC is not an invasive pathogen, other enteric infections and strains of pathogenic *E.coli* can invade via the intestine and cause systemic pathogenesis [206]. IgG antibodies are the predominant antibody classes found in the blood and often mediate the neutralisation and targeting of bacteria for opsonisation by macrophages.

In order to compare the ability of both delivery methods to induce systemic immunity, mice were orally vaccinated as previously described (see 3.3.4) and serum obtained on days 13, 27 and 34 and CFA/I-specific IgG responses measured by end-point ELISA (Figure 3.5.10).

Analysis of serum samples obtained after 3 vaccinations shows no enhancement in CFA/I specific IgG responses in mice vaccinated with FK.ETEC and α-Galcer either in SBC solution or SmPills (Figure 3.5.10 c). However, titres were significantly elevated after 2 rounds of

vaccination (Figure 3.5.10 b) and furthermore, it was determined that delivery by SmPill significantly enhanced the induction of anti-CFA/I IgG in the serum compared to delivery in solution (Figure 3.5.10 d).

3.3.7 – An adjuvant is required to induce potent CFA/I-specific mucosal IgA secretion when utilizing the SmPill to deliver oral vaccines.

Co-administration of α-Galcer enhanced the ability of the FK.ETEC formulation to induce faecal pellet antigen specific antibody responses (Figure 3.5.2 and 3.5.3) which were further enhanced when encapsulated in SmPills (Figure 3.5.6 and 3.5.7).

In order to determine if this effect was mediated exclusively by protection of the antigen by SmPills or if an adjuvant is required, SmPills containing either $3x10^8$ FK.ETEC alone or with 10μg α-Galcer were produced. Mice were orally vaccinated as per figure 3.5.1 with SBC solution alone or one of the two SmPill formulations. Faecal pellets were collected on days 13, 27 and 34 and end-point ELISA performed for CFA/I-specific IgA (Figure 3.5.11) and IgG (Figure 3.5.12).

Mice receiving SmPill formulations of FK.ETEC adjuvanted with α-Galcer had significantly higher CFA/I-specific IgA titres after three rounds of vaccination compared to SmPills with FK.ETEC bacteria alone (Figure 3.5.11 c). Analysis of the anti-CFA/I IgA induction showed a rapid increase in titres after each subsequent vaccination in mice vaccinated with SmPills containing FK.ETEC and α-Galcer versus those with bacteria alone, supported by a significantly higher AUC value (Figure 3.5.11 d).

Similarly, significant increases in faecal CFA/I-specific IgG titres in mice vaccinated with α-Galcer adjuvanted FK.ETEC formulations versus bacteria alone were observed on day 27 and 34 (Figure 3.5.12 b & c). Mice vaccinated with SmPills containing α-Galcer and FK.ETEC

also displayed significantly more rapid induction of CFA/I-specific IgG responses (Figure 3.5.12 d).

3.3.8 – SmPill formulations of FK.ETEC and α-Galcer preferentially promote antigen-specific serum IgG1 titres.

The IgG class of antibodies are the primary isotypes of the systemic antibody repertoire. Depending on the IgG subclass elicited it is possible to have some indication regarding which type of T cell response is predominately induced. IgG2a is indicative of a Th1 response while Th2 cells enhance class switching to IgG1.

In order to examine the nature of the systemic IgG response elicited by oral vaccination with SmPill formulations of FK.ETEC and α-Galcer, a comprehensive investigation of the IgG subclasses induced by this formulation was conducted. SmPills were orally administered to mice as in 3.3.7. Serum obtained on day 34 was investigate for the presence of CFA/I-specific IgG (Figure 3.5.13 a), IgG1 (Figure 3.5.13 b), IgG2a (Figure 3.5.13 c) and IgG2b (Figure 3.5.13 d) by end-point ELISA.

SmPills adjuvanted with α-Galcer elicited significantly stronger serum CFA/I-specific IgG responses compared to FK.ETEC alone in SmPills (Figure 3.5.13 a). A characterisation of this IgG response showed that it was primarily comprised of IgG1, with all 5 mice in the α-Galcer and FK.ETEC group exhibiting antigen-specific IgG1 titres, significantly stronger than in the FK.ETEC group alone (Figure 3.5.13 b). No significant increases in either IgG2a or IgG2b titres were detected in the α-Galcer group (Figure 3.5.13 c & d).

3.3.9 – Significantly higher titres of antigen specific serum IgA are elicited by SmPill formulations containing FK.ETEC and α-Galcer than bacteria alone.

SmPill formulations of FK.ETEC and α-Galcer induced strong faecal CFA/I-specific IgA titres (Figure 3.5.11 and 3.5.12). Serum IgA titres are sometimes used as an indicator of mucosal vaccine efficacy in human trials as antigen-specific IgA^+ ASCs can be detected in the blood after oral vaccination [264]. Studies have shown that circulating IgA-secreting plasma cells in the blood stem from differentiated mucosal B cells that translocate from the mucosa into the systemic immune system following oral vaccination [264, 319-321].

To evaluate if serum IgA titres are elicited by SmPills containing α-Galcer and FK.ETEC, mice were orally vaccinated with SmPills as described in section 3.3.7. Serum was obtained on days 13, 27 and 34 and CFA/I specific IgA (Figure 3.5.14) responses measured by end-point ELISA.

Mice vaccinated with SmPills containing α-Galcer and FK.ETEC exhibited a significantly higher increase in serum IgA titres after 3 rounds of vaccination compared to mice vaccinated with SmPills without α-Galcer (Figure 3.5.14 c). Furthermore, the addition of α-Galcer to the SmPill FK.ETEC formulation elicited a more rapid induction of CFA/I-specific serum IgA titres over the course of the experiment, supported by a significantly higher AUC analysis (Figure 3.5.14 d).

3.3.10 – SmPill formulations of FK.ETEC adjuvanted with α-Galcer induce high titres of local, tissue specific intestinal IgA which is secreted into the intestinal lumen.

Oral vaccination with solutions of FK.ETEC and α-Galcer did not result in an enhancement in CFA/I-specific IgA titres in the intestinal lumen (Figure 3.5.4). When FK.ETEC and α-Galcer were encapsulated in SmPills, these induced marginally higher local small intestinal tissue CFA/I-specific IgA responses than FK.ETEC and α-Galcer delivered in solution (Figure 3.5.8).

In order to determine the benefit of SmPill encapsulation on local intestinal immune responses mice were first administered three doses of PEG to induce mucosal secretions 35 days after the first vaccination (see 3.3.7 for vaccination details). Mice were then sacrificed and perfused. The small and large intestines were dissected out and intestinal washes performed on both the small intestine and large intestine. In addition a 3m segment from the upper Ileum and colon was removed from each mouse. Segments were treated with saponin and end-point ELISAs performed on the supernatants for determination of CFA/I-specific IgA (Figure 3.5.15). In order to account for the dilution of IgA antibodies by the wash and saponin steps CFA/I-specific IgA was adjusted to total IgA concentration (Figure 3.5.15).

Oral vaccination of mice with SmPills containing FK.ETEC and α-Galcer formulations induced a significant increases in antigen-specific IgA titres in both the small intestinal wash (Figure 3.5.15 c) and tissue (Figure 3.5.15 a) compared to FK.ETEC alone. However, the CFA/I-specific titre was found to be higher in the wash (Figure 3.5.15 c) than in the tissue supernatant (Figure 3.5.15 a). CFA/I-specific IgA titres in the large intestinal tissues were significantly elevated in both the wash (Figure 3.5.15 b) and tissue supernatant (Figure 3.5.15 d) in the mice receiving FK.ETEC and α-Galcer formulations versus mice without the

adjuvant (Figure 3.5.15 b & d). However, in contrast to the small intestine (Figure 3.5.15 a & c), large intestinal tissue-supernatant CFA/I-IgA titres were found to be higher than those in the large intestinal wash (Figure 3.5.15 b & d).

3.3.11 – Oral vaccination with FK.ETEC and α-Galcer in SmPills also promotes salivary IgA responses.

SmPills containing FK.ETEC and α-Galcer induced strong local mucosal immune responses in the small intestinal tissue (Figure 3.5.15 a) which were also detected in the large intestinal tissue (Figure 3.5.15 c). Oral vaccination has also been shown to induce both intestinal and vaginal antibody responses in mice [322]. Effective mucosal vaccine formulations as well as infections have been shown to induce both local and disseminated immune responses which can be used as an additional measure of the efficacy of oral vaccination [323].

To determine if FK.ETEC and α-Galcer encapsulated in SmPills can induce antibody responses in other mucosal tissues mice were injected i.p with pilocarpine-HCl to induce salivation 33 days after the initial day of oral vaccination (3.3.7). Saliva was collected and end-point ELISAs performed for CFA/I-specific IgA (Figure 3.5.16). In order to account for the likely difference in the volume of saliva collected per mouse, CFA/I-specific IgA was adjusted to total IgA concentration (Figure 3.5.16).

CFA/I-specific IgA was significantly elevated in the saliva of mice receiving FK.ETEC and α-Galcer SmPill formulations compared to mice vaccinated with FK.ETEC alone in SmPills (Figure 3.5.16).

3.4 – Discussion

ETEC is an enteric infection caused by a pathogenic strain of *E. coli* which accounts for 10-60% of traveller's diarrhoea cases annually [229, 230]. ETEC also accounts for over 10 million cases of diarrhoea in residents of developing countries per annum [231]. This makes ETEC the cause of one fifth of diarrhoeal illnesses in children globally [232] and one of the most frequently identified bacterial enteropathogen in the world and a severe public health hazard in both endemic regions and those susceptible to out-breaks. It is not necessarily a fatal infection since the diarrhoeal illness caused by ETEC is treatable with oral rehydration salts and antibiotics. However, even if adequate medical attention is received, the lasting damage to the intestines caused by the infection can lead to impaired nutrient uptake and so impede development and result in lasting malnutrition in children (section 1.15) [203, 253]. Although immunity to ETEC is acquired after repeated infections, this is in no way an ideal or sustainable solution [253]. Even in travellers to affected regions, ETEC infection can cause long-term intestinal damage after the infection has been resolved. While treatable, ETEC infection is also preventable. Adequate sanitation and fresh drinking water can eliminate ETEC from an endemic area as the main transmission medium for the pathogen is contaminated water and food [226, 227]. However, these solutions are not always economically feasible or implementable in endemic regions or during an outbreak, because these areas often suffer from socio-economic problems including over-crowding, poverty, remoteness and conflict. Currently there is no licenced vaccine against ETEC. While Dukoral®, an oral cholera vaccine can provide transient protection against ETEC Diarrhoea in travellers via cross-reactive intestinal secretory IgA (SIgA) antibodies against CTB/LTB, this protection is very short term in nature, typically lasting only 6-9 months and also has low efficacy in subjects living in endemic regions [293-295]. The WHO has recommended that

vaccines against ETEC be developed, and while several attempts have been made this objective still remains elusive [315].

To date many attempts using oral immunisation with WCK ETEC strains have not elicited satisfactory protection against infection, especially in infants [259]. Several efforts have been undertaken to improve the efficacy of OEVs to elicit protective immunity. As natural immunity to ETEC infection is possible [253], it would make sense to see what components of the ETEC bacterium these immune responses are targeted at in order to determine the best antigen. CFs, which are required by the bacterium to mediate adherence to the host, were found to be amongst the primary targets of natural immunity [232]. Furthermore, mounting protective immunity against CFs could prevent the initial colonisation by the bacterium, thereby limiting its ability to disseminate during diarrhoea and limiting contamination (Holmgren, J.; *personal communication*). This would arrest the cycle of infection where contaminated faecal matter returns viable ETEC organisms into the environment thus adding to the pool of bacteria that can infect new hosts. Therefore a strategy to overexpress CFA/I on non-pathogenic *E.coli* was undertaken in order to generate a candidate OEV (Figure 3.1.5) [310]. In murine studies, this vaccine has been shown to elicit stronger CFA/I-specific IgA and IgG responses following oral vaccination compared to a reference CFA/I expressing ETEC strain (Figure 3.1.5) [310]. CFA/I was chosen as it is present on ETEC isolates from many global regions and thus would provide better global coverage (Figure 3.1.6) [314].

Many oral vaccines require the inclusion of adjuvants in order to elicit protective memory responses in the gut. The most potent mucosal adjuvants are the native and modified heat-labile toxins CT and LT [134]. However the native toxins are much too toxic for use in humans as they would cause diarrhoea (Section 1.11.1). Several mutant derivatives of these toxins have also had their safety profile come under scrutiny by regulatory authorities after failing to meet safety standards for intra-nasal use [153]. In order to find efficacious and safe

oral adjuvants, preliminary screening studies sought to find optimal adjuvants that would elicit immune responses against OVA following oral vaccination (Figure 3.1.7). These studies determined that oral co-administration of TLR agonists with OVA is not effective at eliciting serum antibody responses in solution (Figure 3.1.7 a), which are often used as an indicator of oral vaccine efficacy in humans. Further screening studies identified the glycolipid and iNKT cell activating ligand α-Galcer, which was subsequently shown to be superior to a number of other adjuvants for eliciting serum specific humoral responses following oral vaccination (Figure 3.1.7 b). Furthermore, unlike CT, α-Galcer does not induce fluid accumulation in the small intestine and caecum after oral administration (Figure 3.1.8).

Having selected a potent and tolerated mucosal adjuvant, the ability of α-Galcer could elicit comparable immune responses to the gold standard experimental mucosal adjuvant CT following oral vaccination as part of a novel WCK vaccine was determined. Oral vaccination with FK.ETEC and CT had previously been shown to elicit strong serum IgA and IgG responses in mice (Figure 3.1.5) [310], therefore a study was undertaken to investigate if α-Galcer could potentiate the immunogenicity of the FK.ETEC vaccine in solution. Intestinal SIgA responses have been shown to be one of the most important defence mechanisms against enteric infections in the gut [324]. More importantly natural immunity to ETEC has been linked to strong intestinal SIgA responses [259]. Oral vaccination with FK.ETEC and α-Galcer elicited strong CFA/I specific faecal IgA responses which were comparable to delivery with CT in solution (Figure 3.5.2). Faecal IgA titres have been shown to correlate strongly with responses in the small intestine [317], and co-administration of either α-Galcer or CT elicited stronger titres than FK.ETEC alone. More importantly only mice receiving α-Galcer together with FK.ETEC elicited significantly higher titres compared to mice vaccinated with FK.ETEC alone (Figure 3.5.2).

When ETEC colonises a host, the primary site of infection is in the upper regions of the small intestine [207], therefore an OEV targeted against CFs should be capable of eliciting strong anti-CFA IgA responses at this site to inhibit attachment and colonisation. Both the α-Galcer and CT adjuvanted groups elicited marginally stronger CFA/I-specific IgA titres after oral vaccination than with FK.ETEC alone (Figure 3.5.4). These data may however suggest that faecal IgA titres are a more accurate and reliable read out of mucosal antibody responses than intestinal washes which many dilute the antibodies present in the lumen. Furthermore, faecal pellet titres are representative of "real-time" antigen-specific antibody secretion while PEG may skew the results by enhancing antibody secretion beyond normal levels thus, faecal antibody titres may be a more reliable and accurate measure of antibody titres that exist in the intestine at any given time, and therefore may be a better indicator of a vaccine's potential to prevent attachment of ETEC. A strong induction of a mucosal antibody response combined with the rapid establishment of high titres (Figure 3.5.2 d) after immunisation, could benefit a vaccinated subject during an outbreak by providing a protective immune response after a small number of doses.

A cause for concern is that pathogenic *E. coli* outbreaks in developed countries have also been reported. In Europe the O104:H4 *E.coli* strain caused a large number of hospitalisations and several deaths in 2011. This novel pathogenic *E.coli* strain was transmitted by contaminated vegetables and caught health services by surprise due to its novel phenotype of a enteroaggregative *E.coli* (EAEC) expressing Shiga toxin [325]. Furthermore, in the United States several outbreaks of deadly *E. coli* O157:H7 have become a significant public health hazard [326]. The development of an effective adjuvanted vaccine could allow for the establishment of rapid mass vaccination campaigns in the event of such an outbreak, allow it to be controlled and limit its impact of this on public health facilities.

Although serum antibody responses are not thought to play a significant role in contributing to immune responses in the gut, there is evidence that these can play a role in protection against pathogens at other mucosal surfaces as well as their established role in systemic immunity [117-119]. Serum antibodies can cross the epithelial barriers in both the lung and vaginal tissue but not the intestinal tissue as the former have a "leakier" epithelium due to the nature of their biological function while the intestine has a "tighter" epithelium and therefore requires locally induced and actively transported antibodies to provide protection at the mucosal surface (Holmgren, J. *personal communication*). This may explain why some injectable vaccines such as those against HPV, elicit protection in the form of IgG in the vaginal mucosa [117-119]. However, neither α-Galcer nor CT elicited elevated CFA/I-specific IgG titres in the serum compared to FK.ETEC alone (Figure 3.5.5), suggesting that delivery in solution may be limiting the effectiveness of the α-Galcer and FK.ETEC formulation.

One of the most profound challenges to oral vaccination is the possible destruction of the vaccine formulation in the stomach by acids and in the upper segments of the small intestine by enzymes. While many delivery systems have been developed for oral drug delivery, none have been licenced for use in commercial vaccines. Therefore, developing a delivery system which is capable of improving oral vaccine stability is of paramount importance. The SmPill, a technology developed by Sigmoid Pharma Ltd. is a novel gastro-resistant oral drug delivery system designed for the targeted delivery of pharmaceutical agents to discrete locations in the GIT. Currently the system is in clinical trials for the direct delivery of the anti-inflammatory agent cyclosporine to regions of inflamed intestines in patients with inflammatory bowel disease (IBD). The unique properties of the SmPill make it an attractive system for oral vaccine delivery. Firstly the enteric coating, EUDRAGIT® L 30 D-55 (Evonik Industries), on SmPills allows them to remain stable in the low pH environment of the stomach (Figure 3.1.2

a) [327]. Furthermore, the composition of this coating can be altered in order to target a coated particle to a distinct location in the gut and thereby allow for controlled release of the capsule contents [327]. EUDRAGIT® L 30 D-55 has already been tested for its ability to control the release of an oral cholera micro-particle vaccine composed of WCK *V. cholerae* incorporated into microparticles with encouraging results in rat trials [328]. This study showed that EUDRAGIT® L 30 D-55 did not interfere with antigen stability and led to minimal antigen release under low pH conditions [328]. As the pH was increased, antigen release increased rapidly over seven hours after which antigen release slowed to a more sustained rate [328]. Controlled release of antigen over time has also been shown to aid mucosal vaccination. A number of studies have suggested that the combination of high doses followed by a sustained release of antigen could help overcome the gut's mucosal immune system's predisposition towards tolerance [26, 77, 329]. This has been proposed to be the mechanism behind the efficacy of the oral polio vaccine as the attenuated virus establishes a small local infection yielding both innate stimuli via PAMPs and a reservoir of antigen to establish a memory immune response [15]. A delivery system that has a sustained release capacity may also provide such a reservoir function.

The internal core of the SmPill is composed of a gelatin matrix containing many droplets of an emulsion (Figure 3.1.1 b and 3.1.2 b and c). Upon reaching the upper sections of the small intestine, the enteric-coating of the SmPill pill degrades exposing the gelatin core to the external environment of the gut (Figure 3.1.4). The gelatin is digested by proteolysis and mixing with fluids in the intestine which then releases the emulsion droplets (Figure 3.1.4). The precise mechanism of droplet release and uptake has not yet been documented. The emulsion droplets, which contain the vaccine payload comprises a surfactant, water and the various components of the vaccine (antigens and adjuvant).

However, unlike CT, which is a water soluble protein, α-Galcer is a glycolipid which is much more difficult to solubilize [196]. As the FK.ETEC and α-Galcer components of our candidate vaccine are both hydrophobic and hydrophilic in nature (Figure 1.10), it was deemed prudent to utilise a water-miscible surfactant molecule. We chose the commercially available surfactant Solutol HS-15. Solutol is ~70% lipophilic consisting of polyglycol mono and diesters of 12-hydroxystearic acid and ~30% hydrophilic consisting of polyethylene glycol (Figure 3.1.3). These chemical properties have shown Solutol to form micelle-like structures [313], allowing for the simultaneous solubilisation of both hydrophobic and hydrophilic molecules. Studies have also shown Solutol to increase the bio-availability of compounds while also being safe and well tolerated after oral administration [330].

During the SmPill manufacturing process Solutol is mixed with the vaccine antigens and α-Galcer forming an oil-in-water emulsion. This is then added to liquid gelatin until a uniform distribution is achieved. Following mixing, the gelatin/oil/water emulsion mixture is added drop wise into oil composed of medium chain triglycerides cooled to 4-10°C causing beads to form and solidify. After the formation and gelling, the beads are removed and dried. This expels most of the water from the gelatin matrix but not the droplets of emulsion which are formed by the repulsive effect of the oil which is now thought to contain the vaccine mixture. It is thus plausible for one to speculate that the uptake mechanism of the droplets from the SmPill is similar to that of micelles. These features make the SmPill approach unique as all currently licenced oral vaccines are delivered in solution, while most oral pharmaceutical products are either delivered by solid dosage forms such as pills or solutions as syrups or inside soft capsules. However, no delivery method exists that incorporates both of these attributes is known to this author at the time of writing. The benefits of this method of encapsulation and delivery are many. Firstly, it is possible to manufacture a large number of

accurately dosed pills which required no reconstitution from powders or dosing at the site of administration alleviating the need for trained persons. Furthermore, formulations encapsulated in SmPills may remain stable at room temperature and potentially do not require cold chain storage (Rosa, M. *personal communication*). Currently long term stability and storage condition experiments are being planned to confirm this. The enteric coating on SmPills is not only gastro-resistant but allows for controlled release of the payload at discrete intestinal sites. The gelatin matrix structure, when it degrades also releases the droplets within, increasing the surface area exposed to the vaccine formulation, while simultaneously avoiding the dilution of vaccine contents in the stomach and by intestinal secretions.

With these attributes in mind, it was decided to evaluate the benefit of encapsulating a candidate OEV. Oral vaccination with SmPills containing FK.ETEC adjuvanted with α-Galcer, elicited not only significantly higher titres of faecal IgA after 3 rounds of vaccination (Figure 3.5.6 c) but the establishment of the immune response was significantly more rapid compared with delivery in solution (Figure 3.5.6 d). The enhanced faecal IgA titres induced after three rounds of vaccination with SmPills are not the only benefit over administration of the FK.ETEC in solution as the merits of this delivery system over soluble formulations extend beyond the enhancement of vaccine immunogenicity. One on-going concern when evaluating experimental oral vaccines in mice is the possibility of nasal/bronchiolar contamination during intra-gastric administration (Dougan, G., *personal communication*). Using SmPills as a delivery vehicle completely negates this concern as the capsule and the administration process ensures these reach the stomach without contamination of the nasal/bronchiolar cavity, unlike oral gavage. Therefore the local IgA induced in the intestines of mice receiving SmPills containing FK.ETEC and α-Galcer is entirely the result of an intestinal induction and not due to dissemination of immunity from other mucosal sites

(Figure 3.5.8). The increase in local IgA titre, which was measurable as perfusion clears the intestine of any serum IgA contamination, is indeed due to the enhanced stimulation of local B cell responses as opposed to dissemination of systemic immunity (Figure 3.5.8 a). Although systemic IgG titres were not enhanced when vaccines were delivered in SmPills as opposed to in solution, the rapid induction of systemic immune responses were significantly enhanced by SmPills (Figure 3.5.10).

Vaccine delivery systems may enhance a vaccine's efficacy by shielding the antigen from degradation in the stomach and duodenum, while others include an intrinsic adjuvant effect [312]. The inclusion of an efficacious vaccine formulation in SmPills enhanced its ability to elicit both mucosal and systemic humoral responses (Figure 3.5.6, 3.5.7, 3.5.8 & 3.5.10). However, it is important to determine if this effect was due to the shielding of the formulation or if the SmPill has an intrinsic adjuvant effect itself. If this was the case there may be no need for the adjuvant α-Galcer to be included in the formulation, which would simplify the manufacturing steps involved in generating the vaccine and reducing costs. In the absence of an adjuvant, FK.ETEC alone was unable to elicit strong intestinal IgA titres (Figure 3.5.11). Formulating FK.ETEC with α-Galcer conferred these SmPills with the ability to elicit significantly stronger faecal IgA titres (Figure 3.5.11). Furthermore, the induction of these titres was significantly enhanced by the addition of α-Galcer (Figure 3.5.11 d). These results indicate that an adjuvant is required to elicit immune responses against FK.ETEC when using SmPills as a delivery system. Moreover, at least in the case of FK.ETEC, there seems to be no intrinsic adjuvant effect when using SmPills. This suggests that the efficacy of in the α-Galcer SmPill group is likely due to not only the actions of an efficacious adjuvant but also the enhanced protection of the vaccine payload in SmPills.

An on-going concern with the use of α-Galcer as an adjuvant is that some studies have shown that repeated doses of α-Galcer can induce NKT cell hypo-responsiveness when delivered by

injection [198, 199]. This renders NKT cells unresponsive to additional doses of α-Galcer, causing NKT anergy. However in all studies conducted, α-Galcer elicited both a strong initial induction and significant sustained increase in both mucosal and serum antibody titres, indicating the continuing efficacy of the formulation after each subsequent dose (Figure 3.5.11, 3.5.12 & 3.5.14). Other studies conducted by different research groups support the theory that α-Galcer is an efficacious mucosal adjuvant [188].

In many field trials serum antibody titres against oral vaccine antigens are used as a measure of efficacy [264]. Therefore if a candidate vaccine elicits strong serum antibody titres in animal models it further adds confidence to the potential translation of that vaccine into humans. There are also forms of invasive pathogenic *E.coli* such as meningitis-associated *E.coli* and extra-intestinal pathogenic *E.coli*, therefore if an oral WCK vaccine formulation against one strain can elicit a protective immune response; it may be possible to incorporate additional or different antigens to provide similar protection against other strains. Therefore, if systemic immunity also confers protection at distinct mucosal sites, it may be possible to utilise the oral route together with α-Galcer to elicit protection against pathogens that invade at mucosal sites other than the GIT.

Determining the nature of the IgG subtype induced after successful vaccination provides an indication of the type of T cell responses being elicited, with higher IgG1 titres being indicative of a Th2 response and IgG2a (for BALB/c mice) or IgG2c (for C57BL/6 mice) being the associated with a Th1 response. SmPills loaded with FK.ETEC and α-Galcer elicited very high serum IgG titres against CFA/I (Figure 3.5.13). An analysis found that the predominant subclass of IgG to be IgG1, suggesting that FK.ETEC and α-Galcer elicits a Th2 biased immune response (Figure 3.5.13). IgG1 antibodies have been shown to be capable of crossing over the placenta [331], and most fatalities that are due to ETEC are in infants and young children [233]. The benefits of prenatal vaccines against illnesses such as tetanus have

been well documented [332], and it is not unreasonable to postulate that an efficacious oral vaccine against ETEC might extend the protection elicited in a mother to the neonate.

It has been postulated that many circulating IgA^+ ASCs found in the serum after oral vaccination are in fact of mucosal origin, and so serum IgA titres are a potential readout of a vaccines mucosal efficacy [264]. Serum antigen-specific IgA was significantly enhanced in the FK.ETEC and α-Galcer SmPill vaccinated group after 3 administrations (Figure 3.5.14 c). The inclusion of α-Galcer led to a rapid and significant sustained increase in anti-CFA/I serum IgA titres after 3 subsequent rounds of vaccination (Figure 3.5.14 d). Interestingly, IgA^+ plasma cells have been shown to be able to relocate from the intestine and blood to the mammary glands during lactation [333]. By generating both high titres of serum IgA and mucosal SIgA, vaccines can also protect breastfed infants by providing protective maternal antibodies first in the colostrum and then in the milk [333]. Both these attributes apply to the current work on FK.ETEC and α-Galcer in SmPills, opening up a possibility that these could be used during lactation and breastfeeding to provide enhanced protection for infants via the mother while the mucosal immune system is still developing before oral vaccines against ETEC can be administered to the infant.

Enteric pathogens can cause infection at different sites in the GIT. While ETEC infections occur primarily in the upper segments of the small intestine, other pathogenic *E.coli* strains, such as *E.coli* O157:H7 can cause dysentery which primarily occurs in the large intestine [206]. *E.coli* O157:H7 is a recent strain of enterohaemorrhagic bacteria that spreads primarily through the consumption of under-cooked contaminated meat and can cause haemorrhagic Diarrhoea and kidney failure [326]. Recent outbreaks of such dangerous pathogenic *E.coli* in developed countries have led to several deaths and high profile media attention, as seen during the German *E.coli* O104:H4 outbreak of 2011, which spread throughout Europe and caused a significant public health scare [325]. These outbreaks can be quickly contained by

determining the origin of the outbreak (often the source farm or a food processing plant) and recalling the contaminated products so as to limit their spread and eventual impact on public health. However, the increasing frequency of these outbreaks may necessitate a vaccination strategy in the future to limit the impact of these on public health. Furthermore, several strategies to vaccinate ruminant livestock, the source of *E.coli* O157, have been proposed [334]. Current strategies to deal with *E.coli* O157 in cattle and ETEC in pigs have been to include antibiotics in the food to act as a prophylactic measure. However, increasing concerns about antibiotic resistance and concerns of antibiotics in food have prompted the meat industry to seek alternative means to protect herds and consumers from pathogenic *E.coli*. Vaccination, especially oral vaccination against these threats has the potential to alleviate the need for antibiotics in livestock and may help to economically ease the above mentioned concerns.

Mice vaccinated with SmPills loaded with FK.ETEC and α-Galcer exhibited significantly stronger antigen-specific IgA responses in both the small and large intestine tissue (Figure 3.5.15 a and b). Enhanced IgA responses in both small and large intestinal tissues corresponded with an increase in the luminal IgA in both GIT segments (Figure 3.5.15 c and d). Encapsulation of an oral vaccine formulation in SmPills together with α-Galcer may therefore facilitate the induction of an antigen-specific IgA response along the length of the GIT, providing robust immunity in various locations, thus expanding the oral vaccine potential of the SmPill. However, it must be noted that the CFA/I-specific IgA titre in the small intestinal wash (Figure 3.5.15 c) is higher than that of the tissue (Figure 3.5.15 a), even after adjusting the titres to total IgA concentration. Whereas in the large intestine the tissue CFA/I-specific IgA titre (Figure 3.5.15 b) is higher than in the wash (Figure 3.5.15 d). It is possible that this anomaly is due to the action of the PEG administered prior to the intestinal wash. It is likely that the administration of PEG caused a release of IgA from the LP into the

lumen in the small intestine, thus skewing the balance between tissue and luminal IgA. However, it is unlikely that the PEG would have had sufficient time after administration to exert this effect on the large intestine, and thus the opposite trend is observed in the large intestine.

Many studies have documented that mucosal immune responses induced at one site can extend to other related and even distant sites. This is particularly evident with sublingual vaccination which can elicit immune responses in the intestine [23]. Furthermore, salivary IgA is often used as an indicator of intestinal immune responses following vaccination in humans [335]. However, these secretions can also act as a frontline defensive measure against enteric infection. As most enteric pathogens spread through contaminated food and water, salivary antibodies can engage the pathogens prior to their arrival in the intestine, possibly stopping these from reaching the intestine entirely and/or reducing the number of viable organisms that the intestinal immune system will have to contend with. After 3 rounds of oral vaccination with SmPills containing FK.ETEC and α-Galcer, significantly stronger salivary-IgA responses were detected than in mice receiving SmPills with FK.ETEC alone (Figure 3.5.16).

These studies demonstrated α-Galcer to be an efficacious mucosal adjuvant which does not display the side effects associated with CT. It elicited both stronger local mucosal immune responses but also boosted systemic humoral immune responses. Compared to the gold standard mucosal adjuvant CT, no significant differences were found between CFA/I-specific antibody titres in any of the mucosal and systemic antibody readouts used. The efficacy of this vaccine formulation was further enhanced when incorporated into SmPills. In all readouts utilized, antigen-specific antibody titres induced by FK.ETEC and α-Galcer were higher in SmPills versus in solution, but that this enhancement of antibody responses was α-Galcer dependent. A comprehensive screening of many intestinal, systemic and distant mucosal

readouts revealed that the SmPill formulation of FK.ETEC and α-Galcer was capable of eliciting strong antigen-specific immune responses in all compartments examined.

Furthermore, the extent of the immune potentiation seen in these studies suggests that both α-Galcer in its capacity as a mucosal adjuvant and SmPills as a delivery system have the potential to make a highly positive contribution towards the future success of novel oral vaccines. This approach highlights the need to view the challenges of oral vaccine, namely protection of vaccines from a low gastric pH and proteolysis, the possible dilution of antigens in the GIT, the poor immunogenicity of oral antigens and the lack of mucosal adjuvants as a collective obstacle rather than just individual challenges. Thus, utilising an integrated approach, it is possible to address and overcome many of the challenges associated with designing novel oral vaccines or improving existing ones.

3.5 – Figures

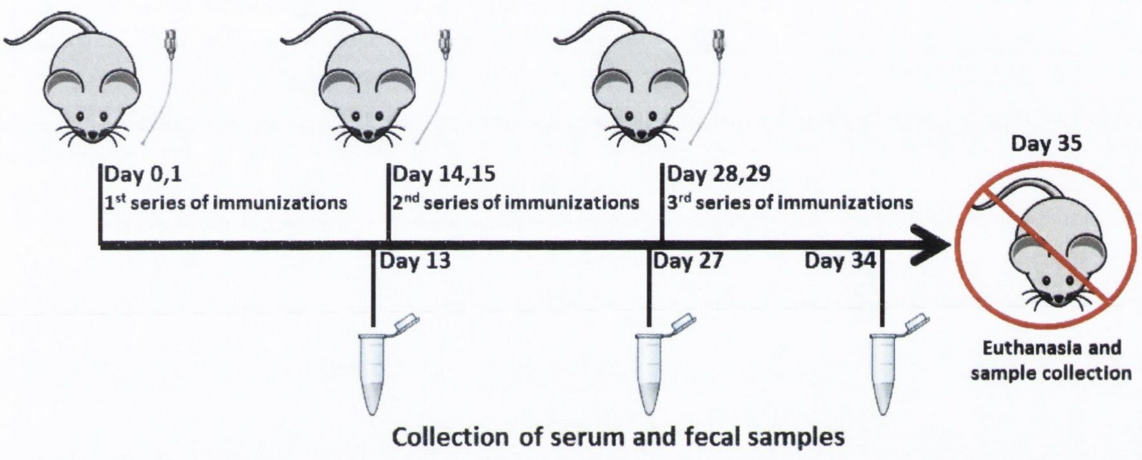
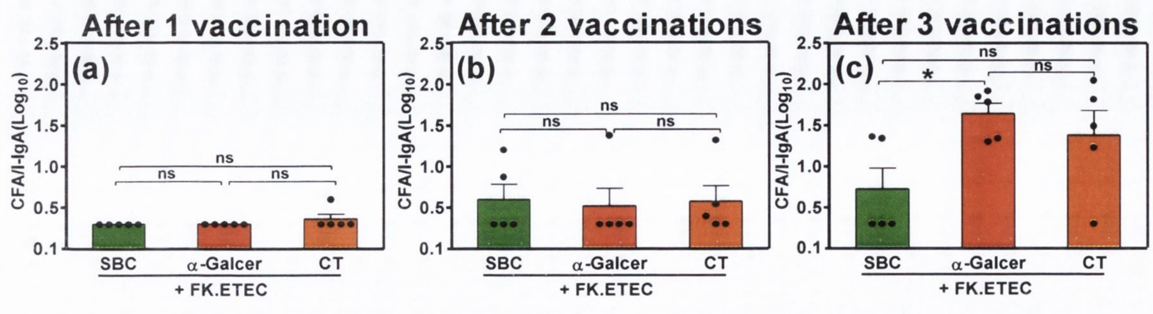


Figure 3.5.1 – Experimental plan for oral vaccine studies in mice involving the evaluation of a novel ETEC whole cell killed vaccine and delivery in SmPills. Mice were orally vaccinated on 2 consecutive days, for 3 rounds, two weeks apart as shown above. The nature of the antigens and adjuvants for each vaccine are detailed in the respective figure legends for each experiment. Fresh faecal pellets were collected together with serum from tail bleeds on days 13, 27 and 34 for analysis of CFA/I-specific antibodies. On day 35 mice were sacrificed by cervical dislocation and tissues were isolated and samples harvested.



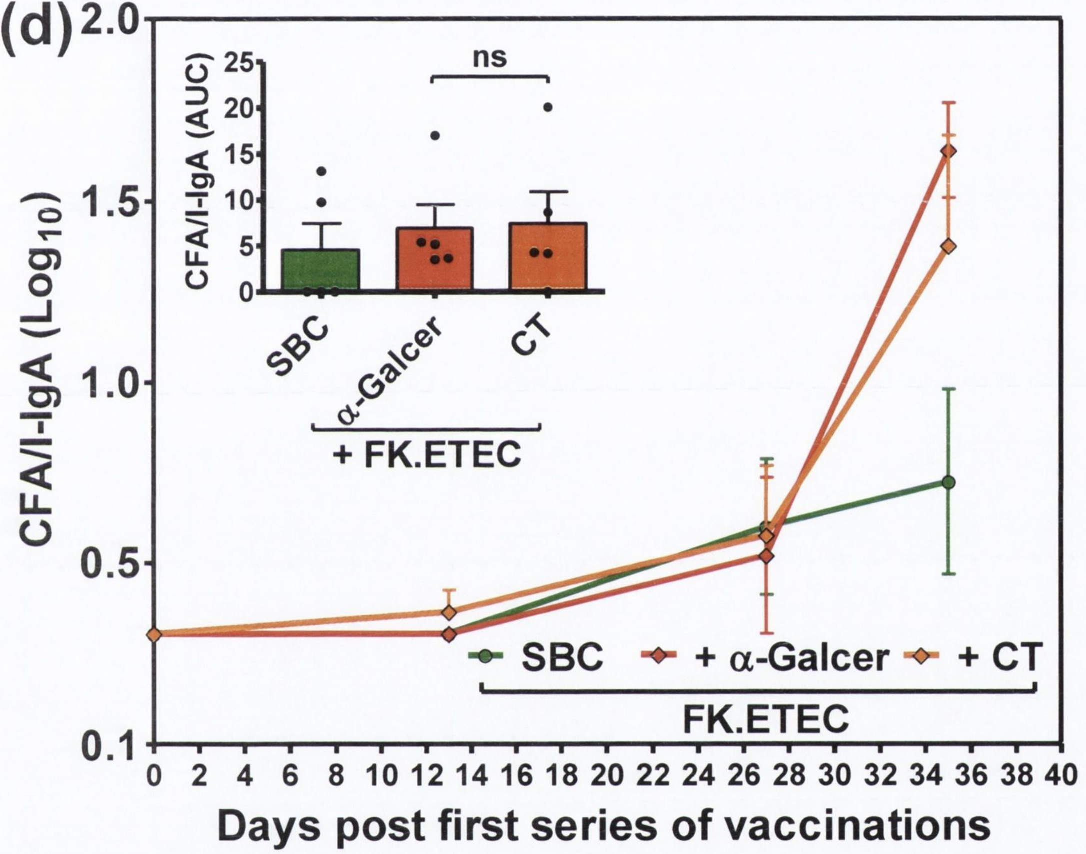
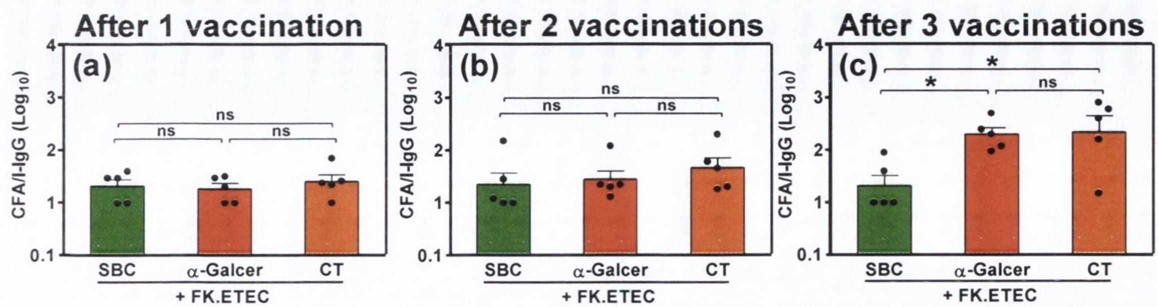


Figure 3.5.2 – α-Galcer is an effective mucosal adjuvant for promoting CFA/I-specific faecal IgA titres after oral vaccination with FK.ETEC. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or FK.ETEC ($3x10^8$ bacteria per mouse) either alone or mixed with CT (10μg) or α-Galcer (10μg) in SBC solution. Faecal pellets were collected on days 13 (a), 27 (b) and 34 (c) and CFA/I-specific IgA antibody titres in the supernatant were determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. SBC (+FK.ETEC) versus CT (+FK.ETEC) versus α-Galcer (+FK.ETEC), * $p<0.05$, ns, not significant. Panel d presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. SBC (+FK.ETEC) versus CT (+FK.ETEC) versus α-Galcer (+FK.ETEC), ns, not significant.



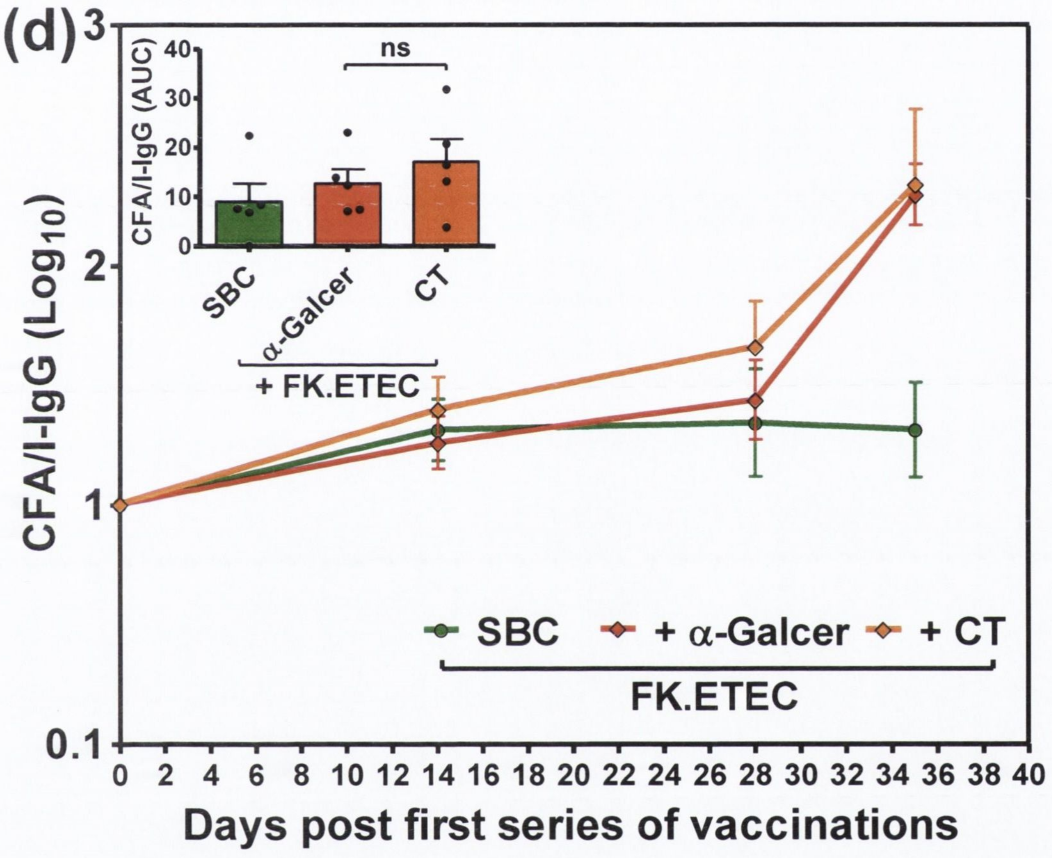


Figure 3.5.3 – Oral formulations of FK.ETEC adjuvanted with either α-Galcer or CT induce high titres of CFA/I-specific faecal pellet IgG. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or FK.ETEC ($3x10^8$ bacteria per mouse) either alone or mixed with CT (10μg) or α-Galcer (10μg) in SBC solution. Faecal pellets were collected and supernatants recovered following centrifugation on days 13 (a), 27 (b) and 34 (c) and CFA/I-specific IgG antibody determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. SBC (+FK.ETEC) versus CT (+FK.ETEC) versus α-Galcer (+FK.ETEC), * $p<0.05$, ns, not significant. Panel d presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. SBC (+FK.ETEC) versus CT (+FK.ETEC) versus α-Galcer (+FK.ETEC), ns, not significant.

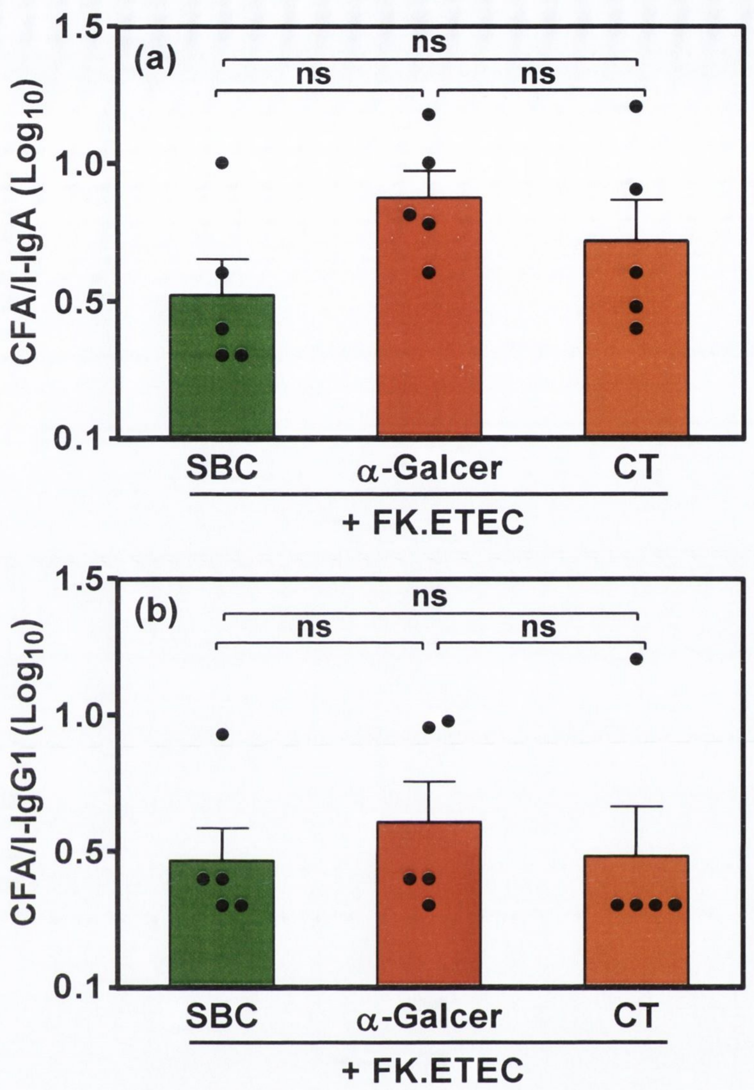


Figure 3.5.4 – Co-administration of α-Galcer with FK.ETEC marginally enhances CFA/I-specific IgA in the small intestinal lumen. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or FK.ETEC ($3x10^8$ bacteria per mouse) either alone or mixed with CT (10μg) or α-Galcer (10μg) in SBC solution. On day 35 mice were given three oral doses of PEG to induce mucosal secretions and then sacrificed by cervical dislocation and following which intestines were removed and wash buffer passed through the lumen. CFA/I-specific (a) IgA and (b) IgG1 antibody titres were determined by end-point ELISA. Panels a and b present mean titres (+ SEM) for 5 mice per experimental group. SBC (+FK.ETEC) versus CT (+FK.ETEC) versus α-Galcer (+FK.ETEC), ns not significant.

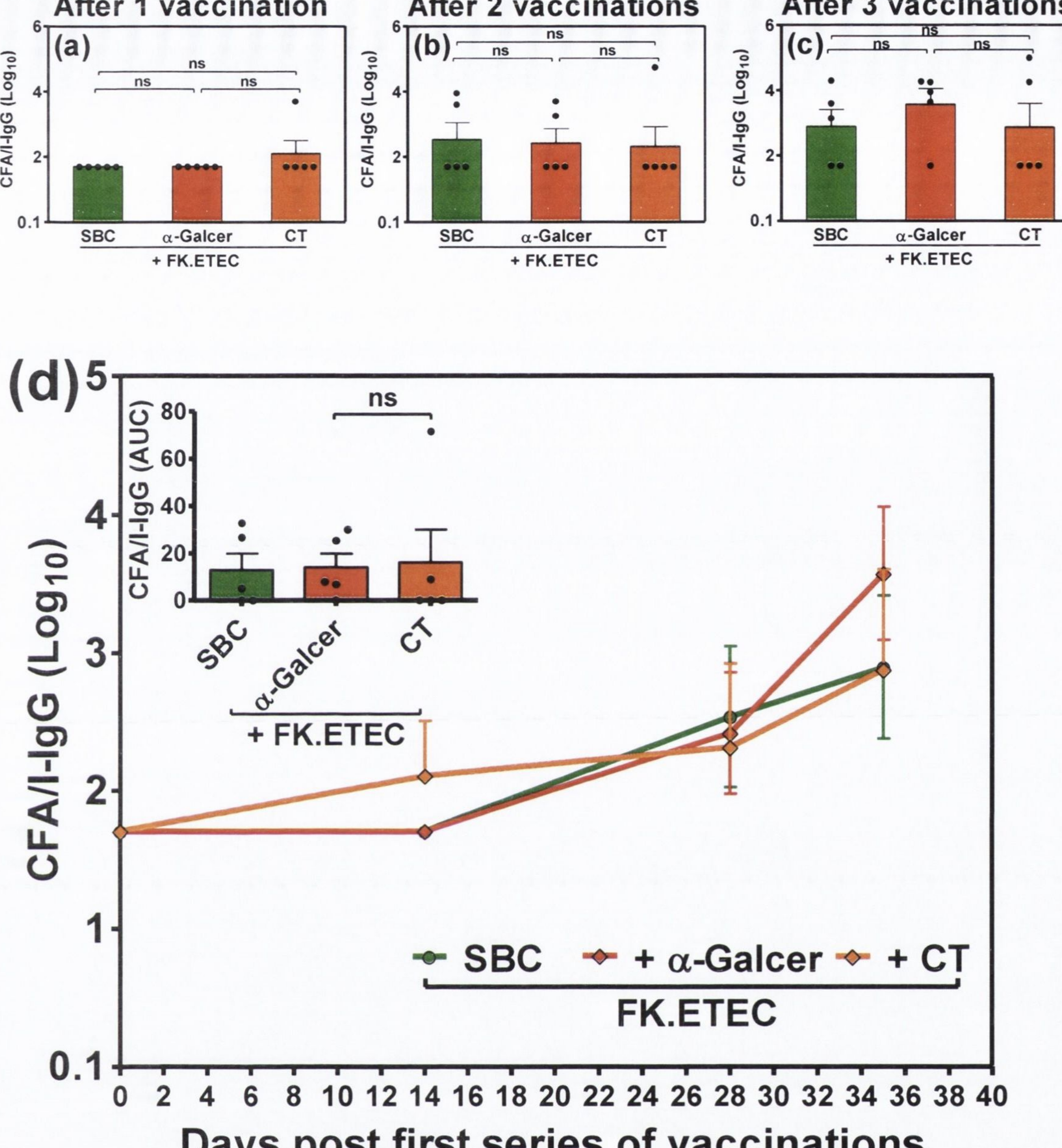
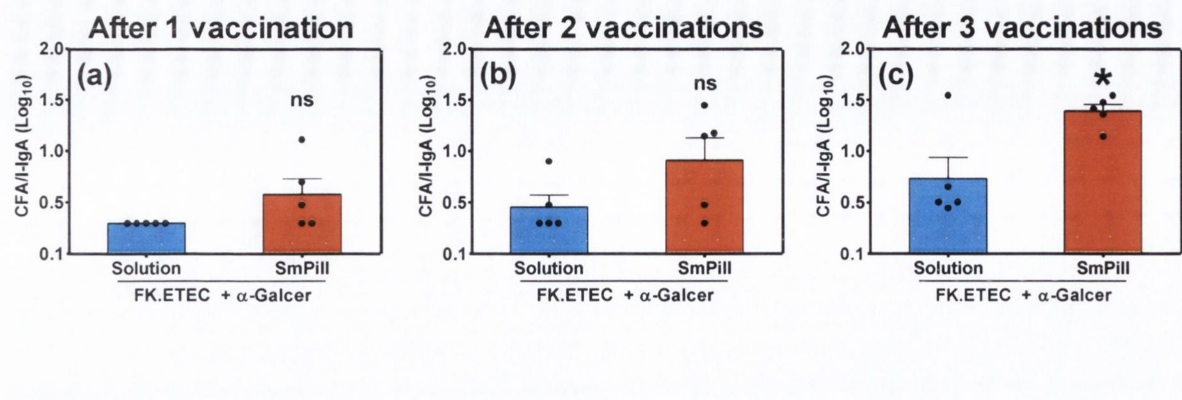


Figure 3.5.5 – No differences in CFA/I-specific serum antibodies were detected after oral vaccination with FK.ETEC adjuvanted with either α-Galcer or CT. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or FK.ETEC ($3x10^8$ bacteria per mouse) either alone or mixed with CT (10μg) or α-Galcer (10μg) in SBC solution. Serum was recovered following tail vein collection on days 13, 27 and 34. Titres were determined by CFA/I-specific end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. SBC (+FK.ETEC) versus CT (+FK.ETEC) versus α-Galcer (+FK.ETEC), ns, not significant. Panel d presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d represents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. SBC (+FK.ETEC) versus CT (+FK.ETEC) versus α-Galcer (+FK.ETEC), ns, not significant.



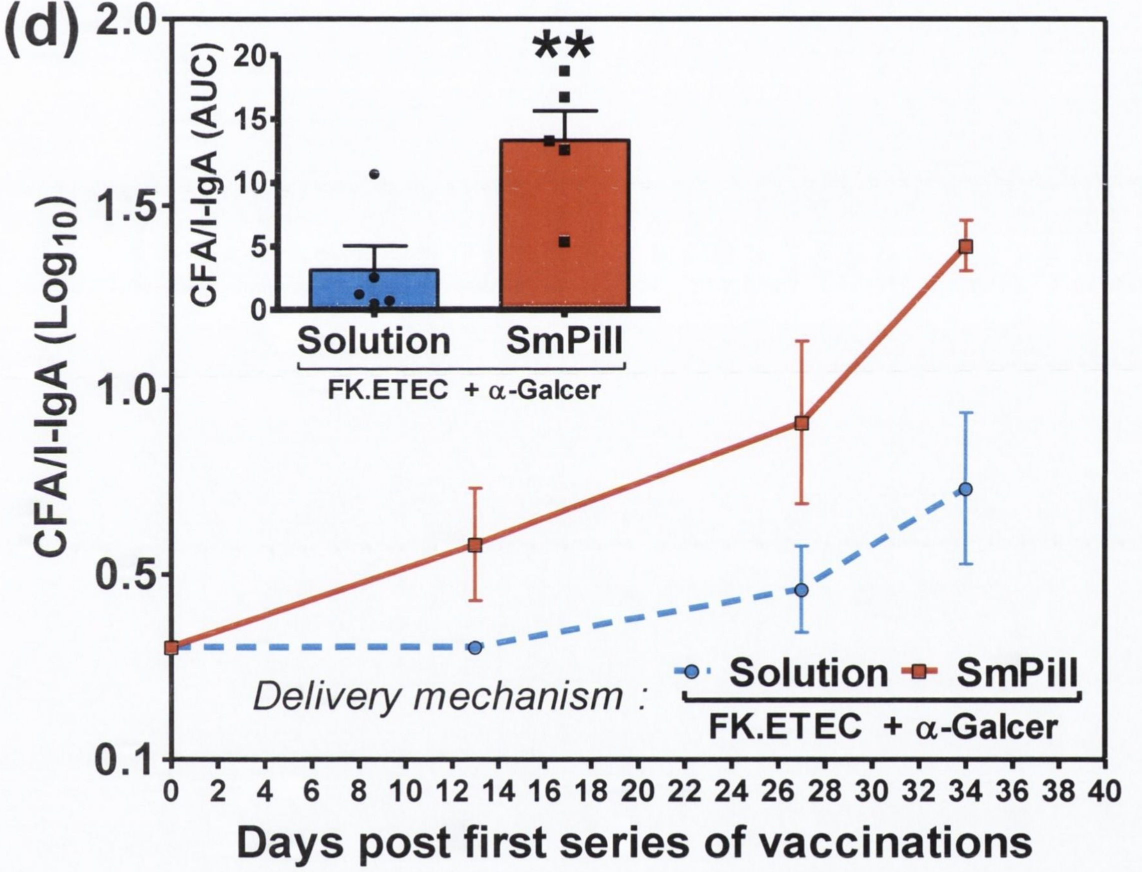
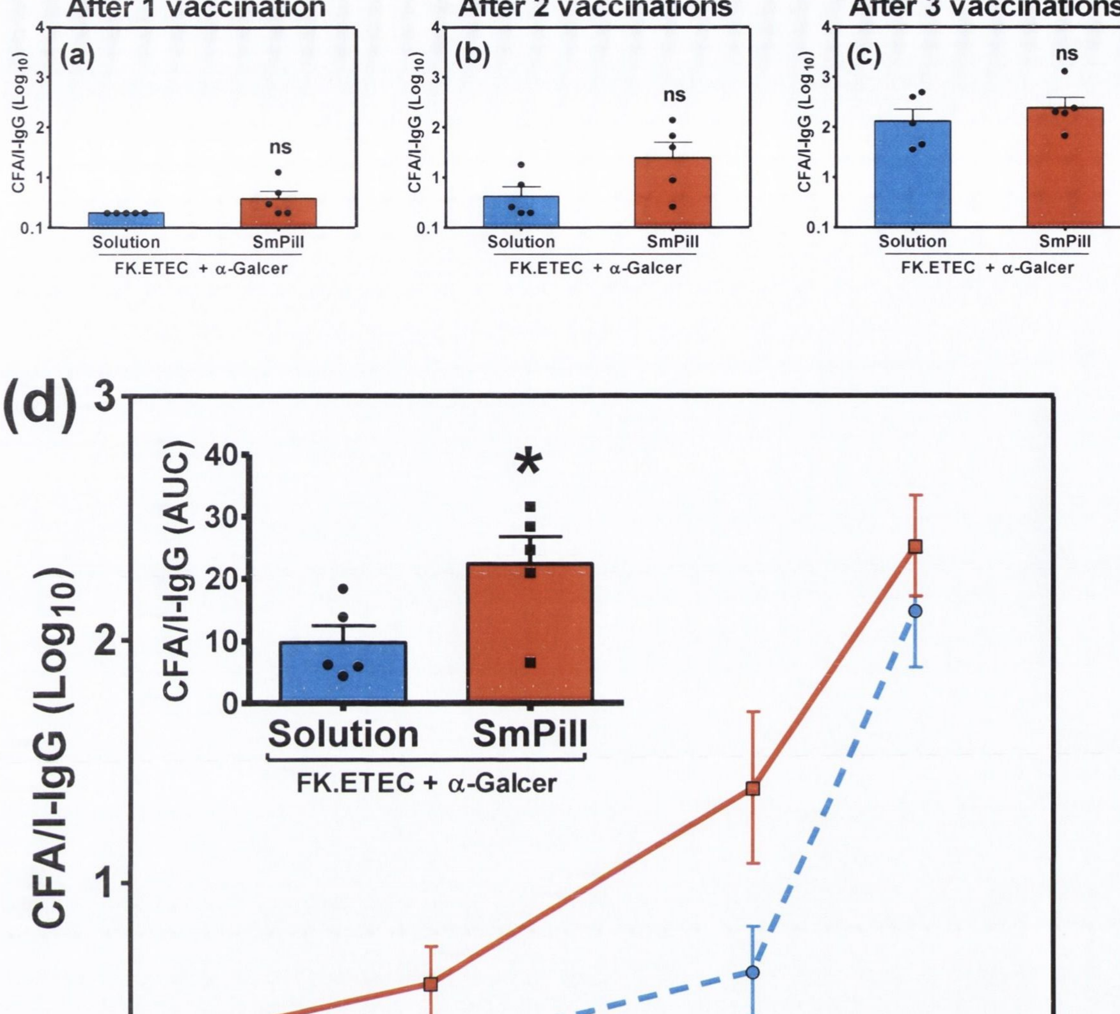


Figure 3.5.6 – FK.ETEC adjuvanted with α-Galcer induces higher titres of faecal IgA when delivered by SmPill than in solution. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or FK.ETEC ($3x10^8$ bacteria per mouse) and α-Galcer (10μg) in SBC solution or encapsulated in SmPills. Faecal pellets were collected on days 13 (a), 27 (b), 34 (c) and CFA/I-specific IgA antibody titres were determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. FK.ETEC and α-Galcer in solution versus FK.ETEC and α-Galcer in SmPills, * $p<0.05$, ns, not significant. Panel d presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d represents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. FK.ETEC and α-Galcer in solution versus FK.ETEC and α-Galcer in SmPills, ** $p<0.01$. (Representative of three independent studies)



Figures 3.5.7 – FK.ETEC adjuvanted with α-Galcer induces a more rapid induce of faecal IgG titres when delivered by SmPill than in solution. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or FK.ETEC ($3x10^8$ bacteria per mouse) and α-Galcer (10μg) in SBC solution or in SmPills. Fresh faecal pellets were collected and supernatants recovered following centrifugation on days 13 (a), 27 (b), 34 (c). CFA/I-specific IgG antibody titres were determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. FK.ETEC and α-Galcer in solution versus FK.ETEC and α-Galcer in SmPills, ns, not significant. Panel d presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. FK.ETEC and α-Galcer in solution versus FK.ETEC and α-Galcer in SmPills, * $p<0.05$. (Representative of three independent studies)

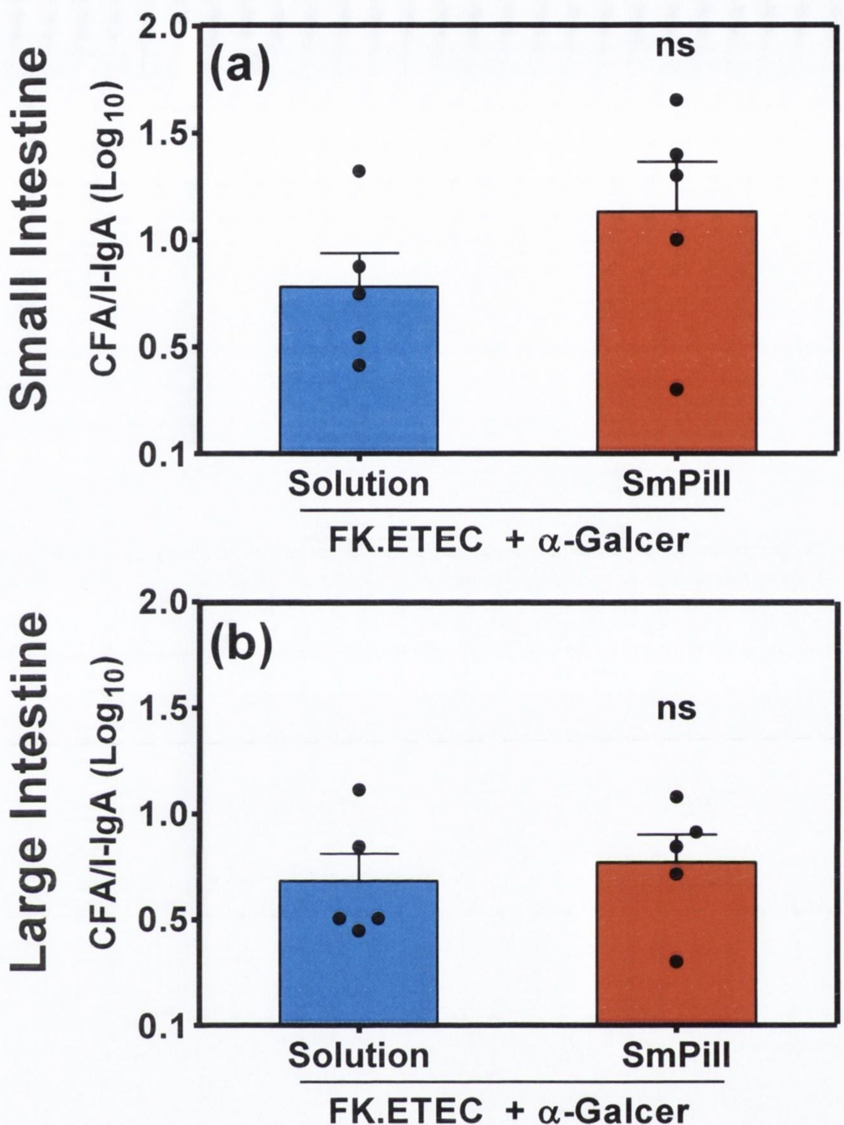


Figure 3.5.8 – Mice vaccinated with SmPill containing FK.ETEC and α-Galcer have marginally higher titres of CFA/I-specific local IgA in the small intestine than those receiving FK.ETEC and α-Galcer in solution. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or FK.ETEC ($3x10^8$ bacteria per mouse) and α-Galcer (10μg) in SBC solution or in SmPills. On day 35, mice were sacrificed by cervical dislocation and perfused with heparin PBS. 3cm of the small and large intestines were dissected out and treated with saponin to extract the inter-tissue antibodies. CFA/I-specific IgA antibody titres in the small (a) and large (b) intestine tissue supernatants were determined by end-point ELISA. Panels a and b present mean titres (+ SEM) for 5 mice per experimental group. FK.ETEC and α-Galcer in solution versus FK.ETEC and α-Galcer in SmPills, ns, not significant. (Representative of three independent studies)

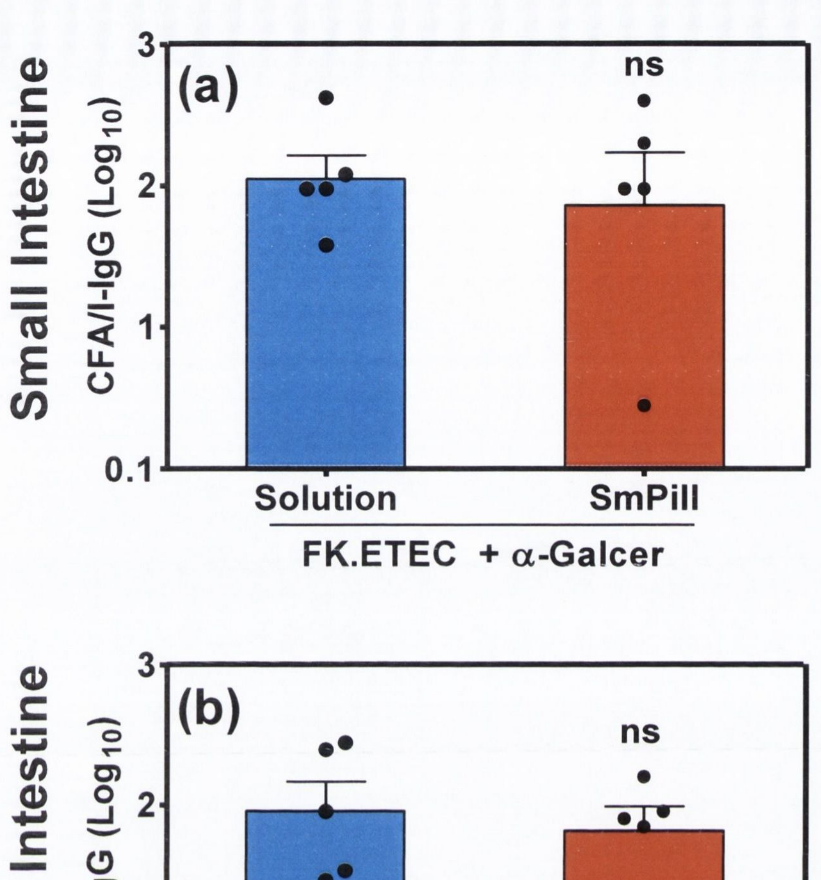
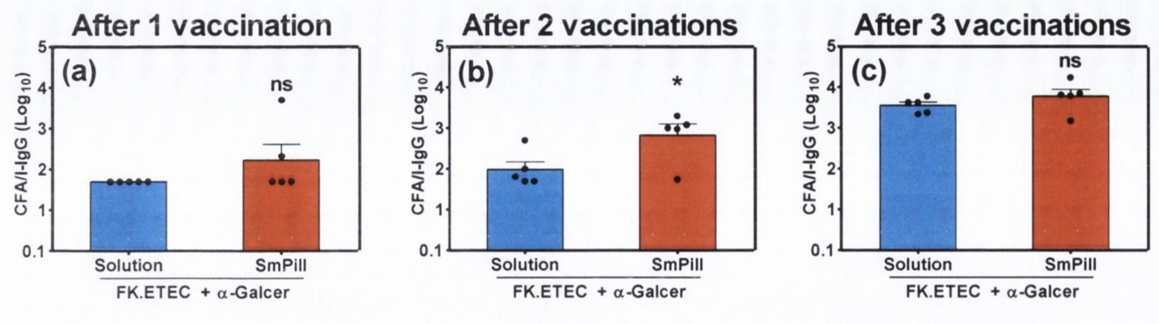


Figure 3.5.9 – Vaccination with SmPill formulations of FK.ETEC adjuvanted with α-Galcer did not induce higher titres of CFA/I-specific local intestinal IgG than in solution. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or FK.ETEC ($3x10^8$ bacteria per mouse) and α-Galcer (10μg) in SBC solution or in SmPills. On day 35, mice were sacrificed by cervical dislocation and perfused with heparin PBS. 3cm of the small and large intestines were dissected out and treated with saponin to extract the inter-tissue antibodies. CFA/I-specific IgG antibody titres in the small (a) and large (b) intestine tissue supernatants were determined by end-point ELISA. Panels a and b present mean titres (+ SEM) for 5 mice per experimental group. FK.ETEC and α-Galcer in solution versus FK.ETEC and α-Galcer in SmPills, ns, not significant. (Representative of three independent studies)



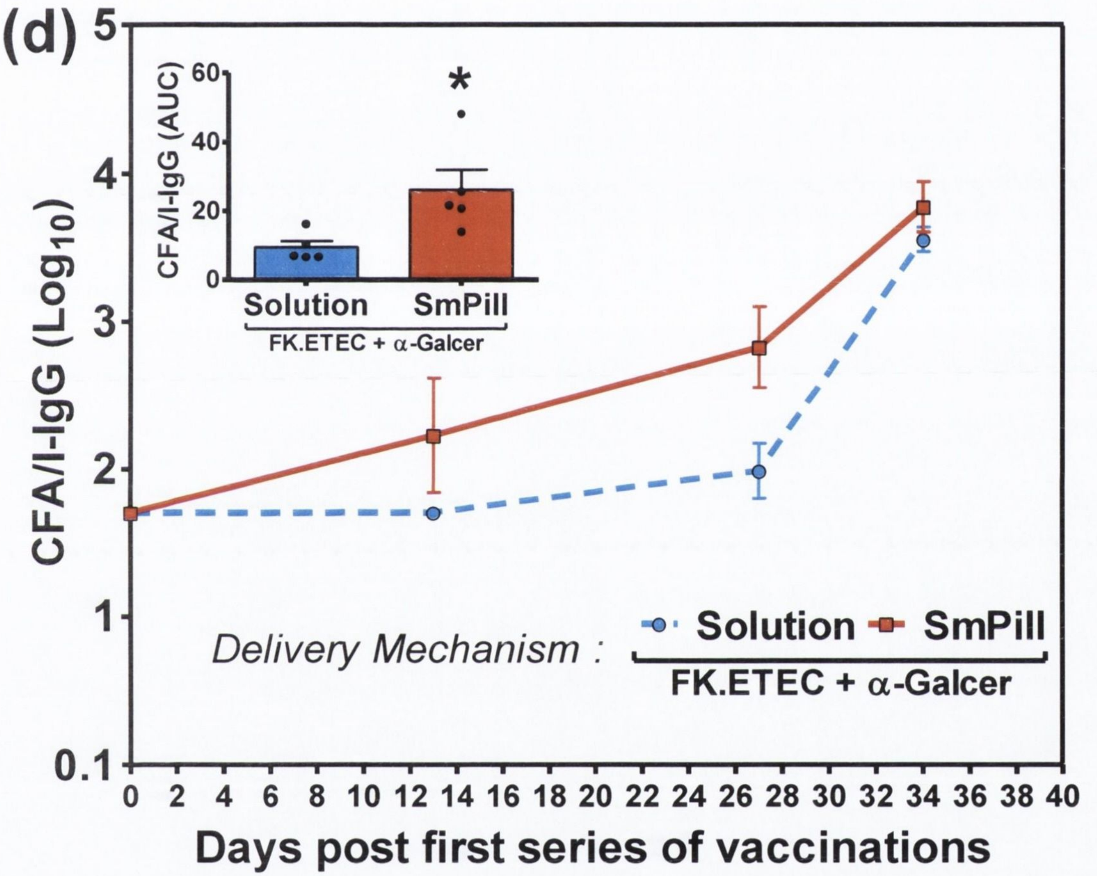


Figure 3.5.10 – Enhanced induction of serum IgG responses was observed after oral vaccination of mice with FK.ETEC and α-Galcer either in SmPills or solution. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or FK.ETEC ($3x10^8$ bacteria per mouse) and α-Galcer (10μg) in SBC solution or in SmPills. Serum was recovered following tail vein collection and centrifugation on days 13 (a), 27 (b) and 34 (c). CFA/I-specific IgG titres were determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. FK.ETEC and α-Galcer in solution versus FK.ETEC and α-Galcer in SmPills, * $p<0.05$, ns, not significant. Panel d presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. FK.ETEC and α-Galcer in solution versus FK.ETEC and α-Galcer in SmPills, * $p<0.05$. (Representative of three independent studies)

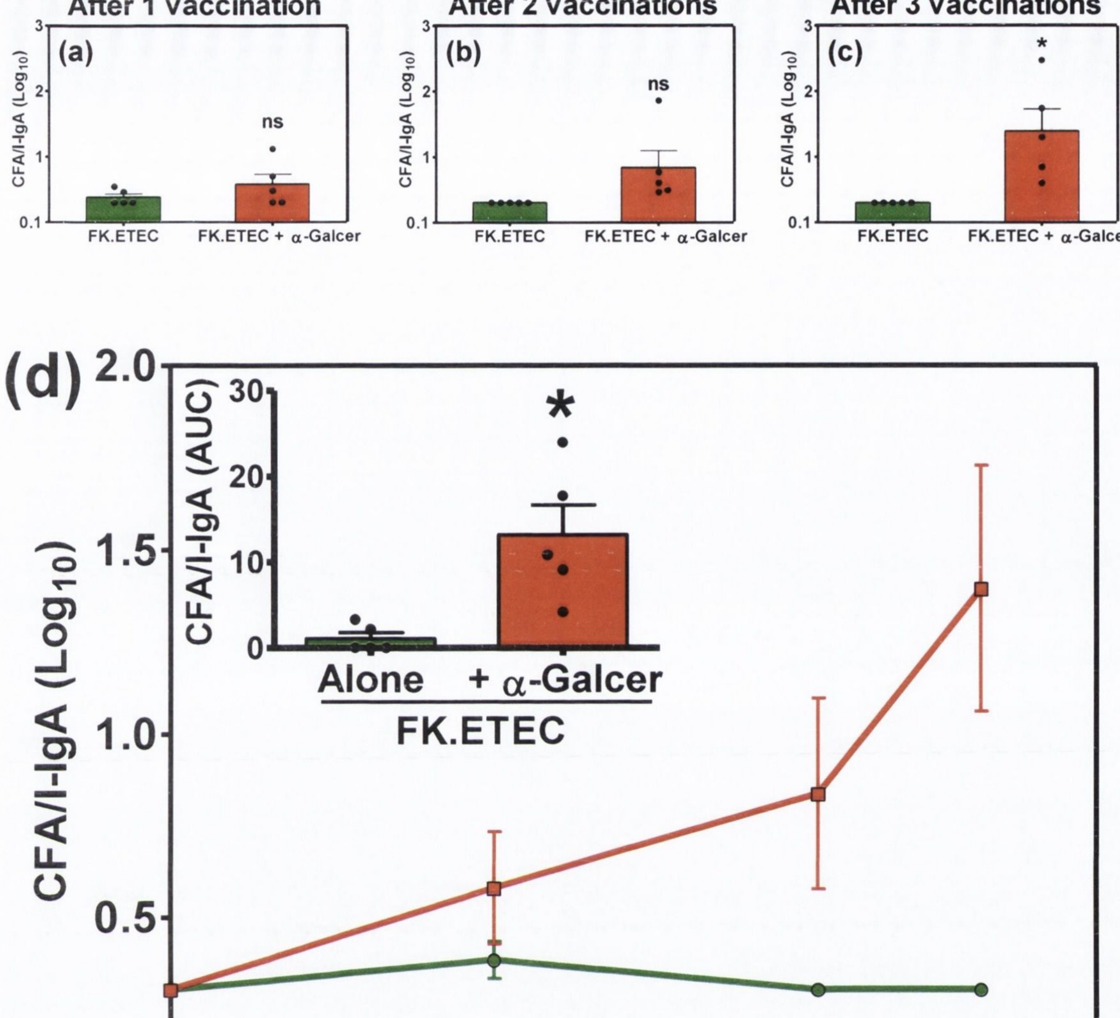
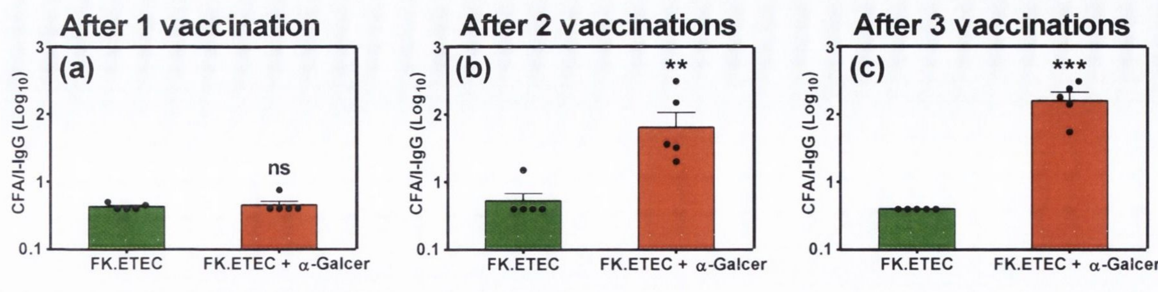


Figure 3.5.11 – α-Galcer is required to induce faecal IgA antibody responses after oral vaccination with SmPills. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or SmPills containing FK.ETEC ($3x10^8$ bacteria per mouse) with or without α-Galcer (10μg). Faecal pellets were collected on days 13 (a), 27 (b) and 34 (c). CFA/I-specific IgA antibody titres were determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. SmPills with FK.ETEC versus FK.ETEC and α-Galcer, * $p<0.05$, ns, not significant. Panel d presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. SmPills with FK.ETEC versus FK.ETEC and α-Galcer, * $p<0.05$, ns, not significant. (Representative of three independent studies)



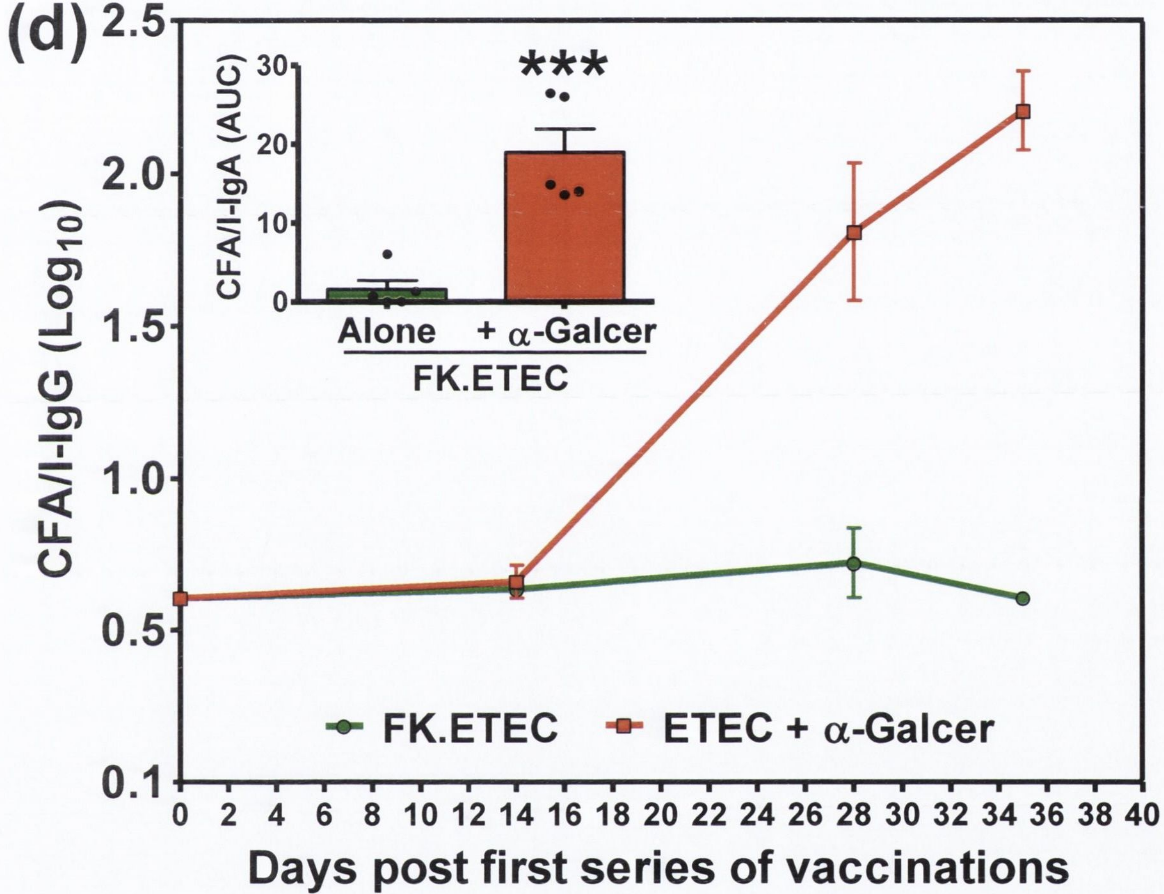


Figure 3.5.12 – Strong faecal IgG titres are only elicited by SmPill containing FK.ETEC when α-Galcer is incorporated. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or SmPills containing FK.ETEC ($3x10^8$ bacteria per mouse) with or without α-Galcer (10μg). Faecal pellets were collected on days 13 (a), 27 (b) and 34 (c). CFA/I-specific IgG antibody titres were determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. SmPills with FK.ETEC versus FK.ETEC and α-Galcer, * $p<0.05$, ns, not significant. Panel d presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. SmPills with FK.ETEC versus FK.ETEC and α-Galcer, * $p<0.05$, ns, not significant. (Representative of three independent studies)

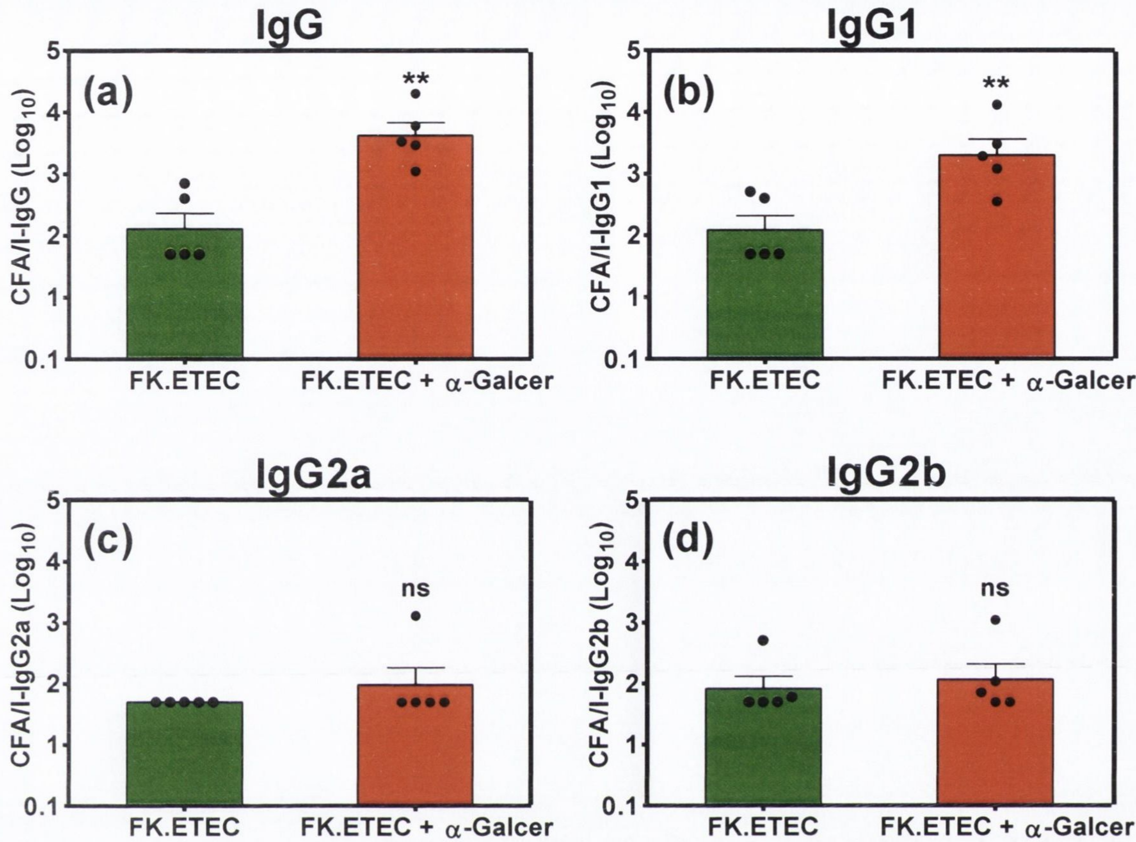
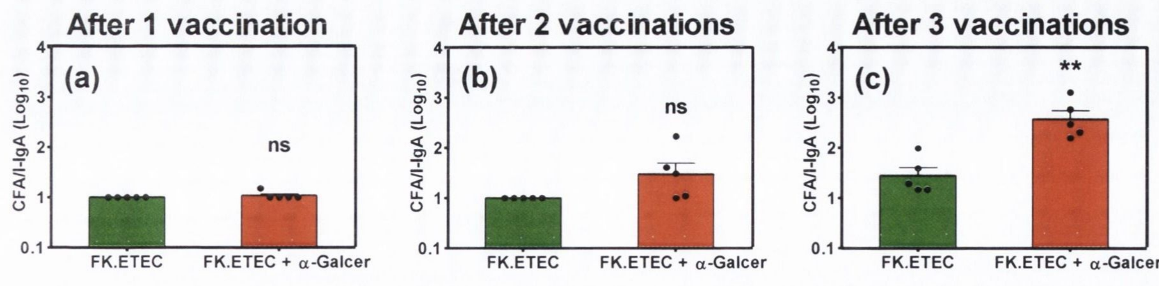


Figure 3.5.13 – IgG1 is the predominant antibody class present in the serum of mice vaccinated with SmPills containing FK.ETEC and α-Galcer. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or SmPills containing FK.ETEC ($3x10^8$ bacteria per mouse) with or without α-Galcer (10μg). Serum was recovered following tail vein collection on day 34. CFA/I-specific IgG (a), IgG1 (b), IgG2a (c) and IgG2b (d) titres were determined by end-point ELISA. Panels a-d present mean titres (+ SEM) for 5 mice per group. SmPills with FK.ETEC versus FK.ETEC and α-Galcer, ns not significant, ** $p<0.01$, ns, not significant. (Representative of three independent studies)



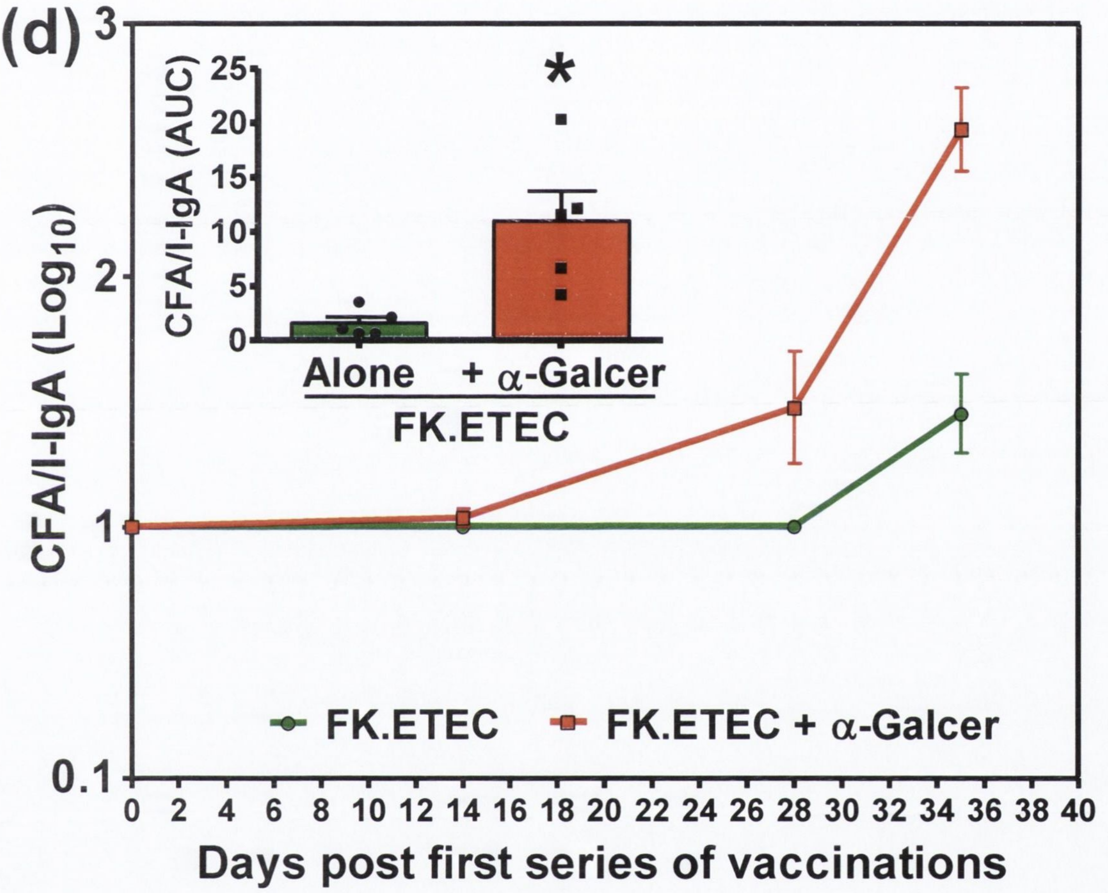


Figure 3.5.14 – Serum IgA titres after oral vaccination with SmPills containing FK.ETEC are significantly increased by the addition of α-Galcer as an adjuvant. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or SmPills containing FK.ETEC ($3x10^8$ bacteria per mouse) either alone or mixed with α-Galcer (10μg). Serum was recovered following tail vein collection on days 13 (a), 27 (b) and 34 (c). CFA/I – specific IgA antibody titres in the serum were determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. SmPills with FK.ETEC versus FK.ETEC and α-Galcer, * p<0.05, ns, not significant. Panel d presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. SmPills with FK.ETEC versus FK.ETEC and α-Galcer, * p<0.05, ** p<0.01, ns, not significant. (Representative of three independent studies)

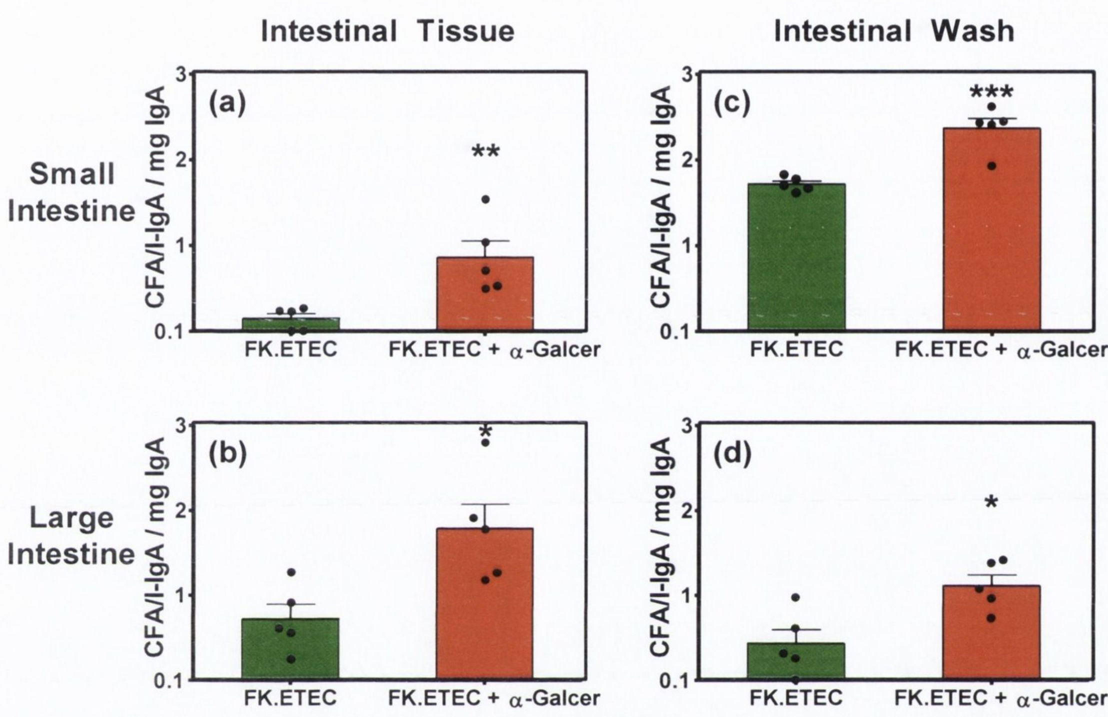


Figure 3.5.15 – The induction of high titres of local intestinal IgA by SmPills containing FK.ETEC is dependent on the incorporation of α-Galcer in the formulation. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or SmPills containing FK.ETEC ($3x10^8$ bacteria per mouse) either alone or mixed with α-Galcer (10μg). On day 35 mice were first administered three doses of PEG to induce mucosal secretions after which they were sacrificed by cervical dislocation and perfused with heparin PBS. Small and large Intestines were removed and wash buffer passed through. 3cm pieces of the small and large intestines were collected and treated with saponin to extract the inter-tissue IgA. CFA/I-specific IgA antibody titres in the small (a) and large (b) intestine tissue supernatants and small (c) and large (d) intestinal wash were determined by end-point ELISA adjusted to total (mg/ml) IgA concentrations. Panels a-d present mean adjusted titres (+ SEM) for 5 mice per group. SmPills with FK.ETEC versus FK.ETEC and α-Galcer, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (Representative of three independent studies)

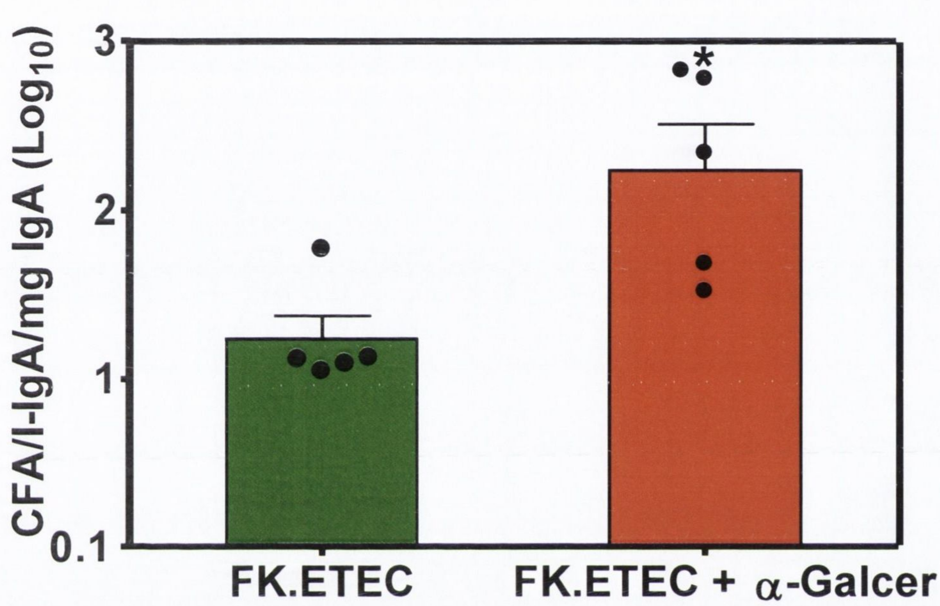


Figure 3.5.16 – Oral (intra-gastric) vaccination with SmPills induces antigen-specific salivary IgA responses. BALB/c mice were vaccinated orally as per figure 3.5.1 with SBC as a control or SmPills containing FK.ETEC ($3x10^8$ bacteria per mouse) either alone or mixed with α-Galcer (10μg). Saliva was obtained after IP injection of pilocarpine-HCl on day 33. CFA/I-specific IgA titres were determined by end-point ELISA adjusted to total (mg/ml) IgA concentrations. Panel a presents adjusted mean titres (+ SEM) for 5 mice per group. SmPills with FK.ETEC versus FK.ETEC and α-Galcer, * $p<0.05$. (Representative of three independent studies)

Chapter Four

Investigating the ability of an integrated oral vaccine strategy to improve the efficacy of vaccines against Cholera

4.1 – Introduction

Cholera is a diarrhoeal disease caused by *Vibrio cholerae* which infects humans through contaminated water and food. The globe is currently experiencing the seventh cholera pandemic and affected regions include much of Southeast Asia, the Indian peninsula, Africa, South and Central America and the Caribbean (Figure 1.27). Although two serotypes, O1 and O139 have been identified in disease isolates, O1 is responsible for approximately 98% of the global disease burden [271]. However, the scale of this burden is difficult to measure as inadequate reporting or misdiagnosis of cases is a problem in developing countries. It is estimated that there are 3-5 million cases annually, resulting in more than 100,000 deaths [211]. While effective rehydration therapy can reduce the fatalities caused by cholera to less than 1%, these are not always available in endemic and out-break regions (Figure 1.28) [210]. Although cholera is rarely endemic in regions with effective sanitation and clean water, it can often spread in the wake of a catastrophe or conflict where such services are disrupted or peoples displaced. In such conditions or in regions where economic and socio-political challenges disrupt public health systems, vaccination can often alleviate the burden of cholera.

Although injectable cholera vaccines were the first to be developed and were in use until the 1970s, these have been abandoned due to a lack of protective efficacy and a poor safety record [284]. Throughout the 70s and 80s knowledge of *V.cholerae* and its induction of immune response greatly advanced, resulting in the appearance of new vaccine candidates. There was also an increased interest in exploiting the oral route to elicit protective immunity against cholera at the primary site of infection [285]. Dukoral® was the first whole cell killed (WCK) oral cholera vaccine (OCV) to be licenced globally by the WHO. It is composed of WCK bacteria of the different serotypes (Inaba and Ogawa) and biotypes (El Tor and

classical) of the O1 strain of *V. cholerae* together with recombinant CTB thus providing bivalent protection against the bacterium and CT. There are two other OCVs, Shanchol™ and Orc-Vax™, containing whole cell killed bacteria of the above mentioned serotypes and biotypes of the O1 strain in addition to bacteria of the O139 serotype which was responsible for cholera epidemics in Asia in the 1990s [285]. However, unlike Dukoral® neither Shanchol™ or Orc-Vax™ contain CTB and thus have no anti-toxic effect [285].

While the above vaccines have shown various degrees of efficacy in clinical and field trials they are by no means perfect. Several factors make these vaccines less than ideal for global distribution and use in very poor countries where they are most needed. The protective effect of these vaccines is generally short term. Anti-cholera protection in subjects vaccinated with Dukoral® drops significantly after each subsequent year, with only 60% of subjects displaying protective efficacy after 2 years and 42% after 3 [292]. Furthermore, the anti-toxic effect of this vaccine only lasts for 3-6 months [292]. In the case of Shanchol™ and Orc-Vax™, protective efficacy lasting 3-5 years has been reported in some trials [300]. In terms of protection against cholera during an outbreak these figures are encouraging, however, with a view to the long term eradication of cholera from endemic region these still leave significant room for improvement.

A second and highly significant challenge to a large scale cholera vaccination effort is the cost involved in vaccine production, storage, distribution and administration. Although in principle oral vaccines provide logistical benefits over injectable ones, the currently available OCVs are by no means as cost efficient as they are required to be. All three currently produced OCVs require the production of individual batches of bacteria expressing the various antigenic targets (e.g. Inaba and Ogawa LPS and the classical and El tor biotypes in addition to the O1 and O139 serogroups) [285]. Furthermore, depending on the vaccine, the different strains of *V. cholerae* also require different methods of inactivation prior to their inclusion in the final

vaccine. In the case of Dukoral® the CTB must also be expressed and purified from a genetically de-toxified strain of *V. cholerae* prior to being added to the final vaccine formulation. In addition, these components must all be produced under good manufacturing practise (GMP) conditions in order to be considered by the WHO for use in humans. This has been an obstacle in global licencing of both Shanchol and Orc-Vax, both of which are striving to meet these challenges [301]. While meeting these goals is important it may also lead to an increase in production costs meaning the goal of bringing these to poor countries might not be met.

While all current oral vaccine formulations require cold chain transport and storage, most regions with endemic and epidemic cholera are among the poorest and most inaccessible in the world. Many such countries lack transportation and utility networks making cold chain storage and transport very expensive. Furthermore, many regions have on-going conflict and displaced populations which add to the expense and difficulty of implementing vaccine programs. All three oral vaccine formulations also require clean water for administration, and Dukoral® requires the addition of a bicarbonate buffer, which neutralises the stomach acid, to protect the CTB subunit from degradation in the stomach which adds an additional challenge and cost. Although Dukoral® has been accepted and licenced by the WHO it's use as an OCV has been primarily restricted to travellers and workers from developed countries who travel to endemic/epidemic regions due to the high cost of the vaccine [285].

Current oral cholera vaccines do not employ a specific delivery system, are administered in solution and elicit protective immune responses in humans [285]. However, novel delivery systems could enhance immunogenicity through protection against stomach acids and enzymatic degradation and delivery to the optimal target tissues. Delivery systems can also positively benefit the storage characteristics of a vaccine by shielding the contents from

environmental factors such as heat and humidity. The addition of suitable adjuvants to the vaccine formulation could also improve the oral efficacy of current and experimental OCVs.

Although there are many challenges to the development of an improved cholera vaccine, several attempts have been made to address these. Encapsulation of WCK *V. cholerae* in a polymer matrix to form microparticles (MPs) is one strategy that has been evaluated by Talavera *et al*. The group incorporated WCK *V. cholerae* bacteria into MPs and then applied a gastro-resistant coating consisting of the enteric polymer Eudragit L-30 D-55 [328, 336]. The coating and polymer not only enhanced the ability of MPs to resist degradation in acidic medium but also provided a controlled release function [328, 336]. Additionally, the group proposed that this mechanism may not only provide enhanced immunogenicity through gastro-resistance and controlled release but also aid antigen uptake via incorporation in MPs [337, 338]. Furthermore, it was also suggested that this approach may sufficiently protect the vaccine from environmental factors to reduce or even alleviate the need for cold chain storage [336]. Other attempts at generating a cold-chain free cholera vaccine include the expression of CTB in rice plants (muco-rice). No differences in antigen content or ability to elicit protective immunity were found between freshly grown muco-rice and that which was stored at either 4°C or 25°C for up to 18 months [339]. However, whether these experimental OCVs will translate into effective human vaccines remains to be determined. In section 3.1.2, the SmPill was introduced as a candidate oral vaccine delivery system. The ability of SmPill to enhance intestinal and systemic antigen-specific antibody responses against a WCK ETEC vaccine in mice was demonstrated in chapter 3 (Figure 3.5.6).

A further obstacle to improving the protective efficacy of OCVs is the lack of safe and effective oral adjuvants. Effective adjuvants could enhance the immunogenicity of antigens and facilitate longer term protection compared to currently licenced OCVs [12]. In section 3.1.4 the challenges of oral adjuvant development and the immunomodulating capacity of the

sphingolipid α-Galcer were introduced. Previous studies have demonstrated that α-Galcer (section 1.12.2) is a potent mucosal adjuvant which is capable of potentiating mucosal immune responses against an experimental FK.ETEC vaccine (see chapter 3). Initially investigated for its anti-tumor properties [196], the immune potentiating effects of α-Galcer have been documented in several studies after both systemic and mucosal administration [188]. In chapter 3, orally administered α-Galcer in solution was shown to increase intestinal IgA responses against CFA/I (Figure 3.5.2). After encapsulation in SmPills, α-Galcer was indispensable for the enhancement of vaccine-mediated intestinal antibody responses (Figure 3.5.11 & 3.5.15). While FK.ETEC over-express CFA/I on a WCK bacterial vector, it was not known if α-Galcer could similarly potentiate anti-LPS mucosal immune responses required for protection against *V. cholerae*.

Herein an attempt was made to address the challenges associated with developing improved OCVs in an integrated manner by utilising the mucosal adjuvant α-Galcer to enhance the immunogenicity of cholera associated antigens and SmPills to deliver these intact through the harsh environment in the stomach.

4.2 – Hypothesis, Aims and Objectives

- Hypothesis -

The limited efficacy of current oral cholera vaccines is due to poor immunogenicity and sub-optimal delivery which can be overcome by a novel integrated oral delivery system and the inclusion of an efficacious oral adjuvant.

- Aims and Objectives -

1. To determine the ability of α-Galcer to potentiate the mucosal immunogenicity of an oral subunit vaccine antigen, CTB.

2. To evaluate the ability of CTB-containing SmPill formulations to confer protection in mice against oral cholera toxin challenge.

3. To determine the potential of orally delivered SmPills containing whole cell killed Inaba-expressing *V. cholerae* co-formulated with α-Galcer to elicit anti-LPS mucosal immune responses.

4. To generate a novel bi-valent SmPill-based oral cholera vaccine containing whole cell killed *V. cholerae* together with CTB co-formulated with α-Galcer.

4.3 – Results

4.3.1 – High doses of CTB delivered in SmPills do not elicit protective immune responses against CT challenge following oral vaccination.

Many approaches to improving the efficacy of OCVs have been attempted. One strategy has been to express a high concentration of CTB in storage bodies in grains of rice called muco-rice [340]. Consuming muco-rice provides high doses of immunogenic antigen which was shown to elicit protection against a murine oral cholera toxin challenge (OCTC) [340]. OCTC involves the oral administration of cholera toxin after a period of starvation, after which the intestines are removed and visually inspected for the presence of CT-induced toxicity (Figure 2.1) [340]. The primary determinants of susceptibility to OCTC are fluid accumulation in the small intestine and caecum (Figure 2.1) [340]. SmPills loaded with a killed whole cell CFA/I overexpressing strain of *E.coli* required the addition of an efficacious oral adjuvant to elicit high intestinal IgA responses (Figure 3.5.11). Due to its ability to bind to and enter a cell via the conserved GM1 molecule, CTB is considered a highly immunogenic antigen which may not require an additional mucosal adjuvant to induce a protective immune response.

In order to determine if SmPills loaded with high doses of CTB could elicit protective immunity against OCTC, 3 batches of SmPills containing 20μg, 40μg or 60μg of CTB were produced. Mice were orally immunised with either SBC alone as a control or with 20μg CTB alone or together with CT as an adjuvant in solution or with one of the doses of CTB in SmPills outlined in figure 4.5.1. Faecal pellets were collected on day 41 and CTB-specific IgA titres determined by ELISA (Figure 4.5.2 a). On day 42 mice were orally challenged with CT and 16 hours after challenge mice were euthanized by CO_2 asphyxiation and the intestines dissected out and photographed (Figure 4.5.2 c). In each case, the caecum was separated from the intestine and weighed. Caeca were then dried for 1 week at 37°C and weighed again. The

percentage of fluid lost to evaporation was calculated and diarrhoea was determined by a higher percentage of fluid in the caecum (Figure 4.5.2 b).

Delivering CTB by SmPill increased the faecal IgA titre in comparison to CTB in solution (Figure 4.5.2 a). However, there was only small dose-dependent increases in titre observed in mice receiving SmPills (Figure 4.5.2 a). No dose of CTB in SmPill elicited responses close to the magnitude of the positive control CT and CTB (Figure 4.5.2 a). Furthermore, only mice receiving CT and CTB in solution were protected against OCTC (Figure 4.5.2 c). Mice immunised with SmPills containing CTB all had high percentages of fluid in their caeca after challenge, which is indicative of diarrhoea (Figure 4.5.2 b). Only when immunised with CT and CTB were mice protected from fluid accumulation in the caecum (Figure 4.5.2 b). However, it was observed that mice vaccinated with SmPills containing 40µg or 60µg CTB had significantly lower fluid content in their caeca than those vaccinated with 20µg CTB (Figure 4.5.2 b)

4.3.2 – CTB can be accurately loaded into SmPills.

SmPills containing high doses of CTB did not elicit protective immunity (Figure 4.5.2) suggesting that it is necessary to include a mucosal adjuvant in the formulation. However, to ensure efficacious and reproducible results it was important to ensure that SmPills can be accurately loaded with subunit antigens.

In order to address this issue, SmPills from the same batch as used in figure 4.5.2 were dissolved in PBS and mixed with SDS-PAGE sample buffer. Samples were run on a 15% gel by SDS-Page and stained with Coomassie Blue. CTB in SmPill extracts was compared to 10, 20, 60 and 100µg CTB protein standards (Figure 4.5.3). Bands are representative of protein concentrations prior to dilution and running on the gel.

CTB bands corresponding to SmPills loaded with 20μg CTB have a similar appearance on the gel to the 20μg CTB standard (Figure 4.5.3). SmPills containing 60μg CTB also corresponded to the 60μg standard on the gel, while 40μg CTB SmPills samples appeared darker than 20μg CTB but lighter than the 60μg standard (Figure 4.5.3). No band at 12kDa was detected in the case of SmPills containing ovalbumin (OVA) confirming that there was no interference from other capsule components (Figure 4.5.3).

4.3.3 – The inclusion of a potent mucosal adjuvant generates SmPills that can induce strong faecal IgA responses after oral vaccination.

Oral delivery of SmPills containing CTB alone did not elicit protection against OCTC (Figure 4.5.2). This however, was not due to insufficient loading (Figure 4.5.3). Some manufacturing processes can damage subunit antigens, which are often more susceptible to degradation than WCK antigens due to their delicate nature.

To examine whether the inclusion of a mucosal adjuvant in SmPills would generate an effective mucosal immune response and to determine the ability of protein antigen and labile adjuvants to be incorporated intact, SmPills containing 20μg of CTB alone or together with 10μg CT were manufactured. Mice were orally immunised with either SBC alone as a control or with 20μg CTB alone or with CT as an adjuvant in solution or encapsulated in SmPills as per figure 4.5.1 Faecal pellets were collected on day 27 (a) and 41 (b) and CTB-specific IgA titres determined by end-point ELISA (Figure 4.5.4).

Mice vaccinated with SmPills containing CT and CTB had lower titres of faecal IgA compared to mice given the same formulation in solution after 2 rounds of vaccination (Figure 4.5.4 a). However, after 3 rounds of vaccination these titres increased and no significant difference was found between administering CT and CTB either in solution or in SmPills (Figure 4.5.4 a and b). Administration of CTB alone either in solution or in SmPills

failed to elicit strong faecal IgA responses at any time point (Figure 4.5.4 c). A significantly lower induction of CTB-specific IgA responses was observed in mice receiving CT and CTB by SmPill versus in solution (Figure 4.5.4 c).

4.3.4 – Vaccination with CT and CTB in SmPills effectively promoted antigen-specific serum antibody responses.

While many methods exist to determine the protective efficacy of an oral vaccine in animal models, in humans these are much more restricted. One accepted correlate of mucosal vaccine efficacy in humans is serum antigen specific antibodies to a vaccine antigen [323]. Therefore, if a candidate vaccine is capable of eliciting serum IgA and IgG responses in mice, this may be an indication of its ability to do so if trialled in humans.

To determine if the SmPill manufacturing processes attenuated the ability of CT and CTB to elicit serum responses, mice were orally immunised as described in 4.3.3. Serum was obtained on day 41 and antigen-specific IgA (a), IgG1 (b) and IgG2a (c) titres determined by end-point ELISA (Figure 4.5.5).

No significant differences were detected in serum antigen-specific IgA titres between mice receiving CT and CTB in solution or SmPills (Figure 4.5.5 a). Likewise IgG1 and IgG2a titres were not significantly different between mice receiving CT and CTB in SmPills compared to solution (Figure 4.5.5 b and c).

4.3.5 – The ability of CT and CTB to promote protective immunity against oral cholera challenge is maintained when CT and CTB are encapsulated in SmPills.

While the ability of an oral vaccine to elicit both faecal IgA (Figure 4.5.4) and serum antibody (Figure 4.5.5) responses are both markers of a vaccine efficacy, the most effective measure of

a vaccines protective efficacy is its ability to render a host immune to a challenge with the disease causing agent.

To examine if the encapsulation of CT and CTB would reduce their ability to induce protection against OCTC the following study was conducted. Mice were orally immunised as described in 4.3.3. On day 42, mice were orally challenged with CT and 16 hours later mice were euthanized by CO_2 asphyxiation and the intestines dissected out. The intestines were photographed (Figure 4.5.6 a) after which the caecum was separated from the intestine, weighed, dried for 1 week at 37°C and weighed again. The percentage of fluid lost to evaporation was calculated. The presence of diarrhoea was determined by the accumulation of fluid in the intestines as seen in photographs (Figure 4.5.6 a and b) and a higher percentage of fluid in the caecum (Figure 4.5.6 c).

Mice receiving CT and CTB both in solution and in SmPills were equally protected against OCTC (Figure 4.5.6 a and b). In contrast mice vaccinated with SBC or CTB in solution or SmPills were not protected against oral cholera challenge and had large volumes of fluid in both the small intestine and caecum (Figure 4.5.6 a and b). When percentage fluid levels in the caecum were measured, no significant differences were found between unchallenged SBC control mice or mice vaccinated with CT and CTB in either solution or SmPills (Figure 4.5.6 c). Mice vaccinated with CTB in solution or in SmPills had high fluid levels in the caecum, consistent with a lack of protection from cholera toxin challenge similar to unvaccinated challenge controls (Figure 4.5.6 c).

4.5.6 – A method to characterise the incorporation of whole cell killed antigens in SmPills and determining antigen stability.

When SmPills were produced containing the subunit antigen CTB it was possible to qualitatively determine antigen loading on SDS-PAGE gels stained with Coomassie Blue

(Figure 4.5.3). However, when using WCK bacteria expressing an LPS-antigen SDS-PAGE is not an optimal method to qualify accurate loading into SmPills. Therefore it was assessed whether it was possible to determine bacterial loading into SmPills and to ensure that the LPS-antigen was intact by dot blot analysis.

SmPills containing $2.5x10^8$ Inaba LPS expressing, detoxified (JS1569) *V. cholerae* with or without 10μg αGalcer were produced. SmPills were dissolved in PBS and supernatants assayed via dot blots. A reference dot containing ~$1x10^9$ JS1569 *V.cholerae* from the same batch used to produce SmPills was also applied to the plate. Antibodies against the common *V.cholerae* antigen or against Inaba-LPS were applied to dot blots.

SmPills containing $2.5x10^8$ JS1569 bacteria both with and without α-Galcer tested positive for *V.cholerae* bacteria and appeared at similar intensities suggesting even loading across both formulations (Figure 4.5.7). Both SmPill formulations also contained much lower bacterial numbers compared to control dots containing ~$1x10^9$ cells of the same batch as determined by lower exposure intensity (Figure 4.5.7). WCK JS1569 cells isolated from the SmPills also appeared to contain intact Inaba-LPS, which appeared in lower quantities to reference controls (Figure 4.5.7).

4.3.7 – SmPills enhance the ability of a novel oral whole cell killed *V. cholerae* vaccine to elicit faecal IgA responses.

When formulations of CFA/I overexpressing *E.coli* adjuvanted with α-Galcer were encapsulated in SmPills this potentiated the ability of these to induce mucosal immune responses (Figure 3.5.6). While CFA/I is a protein antigen, most vaccines against *V. cholerae* are targeted against the O-antigen of the LPS molecule. Therefore the desired vaccine-elicited immune response against *V. cholerae* is vibrocidal anti-LPS IgA.

In order to determine if SmPills could enhance anti-LPS intestinal IgA responses SmPills containing an Inaba LPS expressing, detoxified *V. cholerae* (JS1569) strain with or without α-Galcer were produced. Mice were immunised with SBC alone as a control or with 2.5×10^8 JS1569 either with or without α-Galcer in solution or with the SmPill formulations outlined above and as per figure 4.5.1. Faecal pellets were collected on day 13 (a), 27 (b) and 41 (c) and Inaba LPS-specific IgA titres determined by ELISA (Figure 4.5.8).

After 3 rounds of oral vaccination with JS1569 and α-Galcer in solution Inaba LPS-specific intestinal IgA responses were detected (Figure 4.5.8 c). In contrast, vaccination with JS1569 alone, either in solution or SmPills did not induce detectable responses at any time point during the course of the study (Figure 4.5.8). When encapsulated in SmPills, JS1569 with α-Galcer elicited significantly stronger antigen-specific intestinal IgA responses after 2 rounds of vaccination compared to delivery in solution (Figure 4.5.8 b). Furthermore, a more rapid induction of faecal IgA responses was observed ((Figure 4.5.8 d).

4.3.8 – SmPill delivery of whole cell killed *V. cholerae* with α-Galcer enhances anti-LPS IgA responses in the small intestine.

Local IgA-mediated immunity is one of the most important defence mechanisms against enteric infections in the GIT and immunity to *V. cholerae* has been shown to depend on antibody responses against the O-antigen of the LPS molecule [341].

Mice were immunised as described in 4.3.7. On day 42, mice were euthanized by cervical dislocation and perfused with heparin PBS after which the intestine was dissected out, 3cm of the small intestine at the beginning of the ileum was removed for saponin extraction. Inaba LPS-specific IgA responses in the tissue supernatant were determined by end-point ELISA (Figure 4.5.9).

Local tissue LPS-specific IgA titres elicited by vaccination with JS1569 and αGalcer were not significantly increased by encapsulation in SmPill compared to administration in solution (Figure 4.5.10). Furthermore, oral vaccination with JS1569 and αGalcer in SmPills elicited significantly higher titres compared to mice vaccinated with SmPills without αGalcer (Figure 4.5.10).

4.3.9 – Oral vaccination with *V. cholerae* and α-Galcer in SmPills does not enhance serum anti-LPS antibodies.

To investigate if anti-LPS serum antibody titres can be detected after oral vaccination, Mice were immunised as described in 4.3.7. On day 41 serum was recovered and LPS-specific IgA (Figure 4.5.10 a) and IgG1 (Figure 4.5.10 b) in the serum were determined by end-point ELISA.

After 3 rounds of vaccination with JS1569 and αGalcer either in SmPills or in solution, no significant differences in serum anti-LPS IgA (Figure 4.5.10 a) or anti-LPS IgG1 titres (Figure 4.5.10 b) were detected.

4.5.10 – α-Galcer and CT differentially enhance faecal IgA responses to particulate and soluble antigens.

The most effective licenced OCV currently is Dukoral® which incorporates both recombinant (rCTB) and 3 strains of WCK *V. cholerae*. However, the high cost associated with this vaccine has limited its application in mass vaccination programs in endemic regions. In order to address the issue of cost, a single strain of *V. cholerae* expressing both Inaba and Ogawa LPS, the MS1346 strain, was developed to help overcome this barrier [307]. α-Galcer was shown previously to enhance the mucosal immune responses against WCK *V. cholerae* expressing Inaba LPS (Figure 4.5.8) and against CFA/I expressed on *E.coli* (chapter 3).

In order to determine if α-Galcer could elicit a comparably enhance immune response to a mixture of antigens, mice were immunised orally as per figure 4.5.1 with PBS as a control. Treatment groups included mice vaccinated with CTB (27.5µg per mouse) and MS1346 *V.cholerae* ($3x10^8$ Bacteria per mouse) either with or without CT (10µg) or α-Galcer (10µg) as an adjuvant. On days 13, 27 and 41 faecal pellets were collected and CTB-specific (Figure 4.5.11 a, b, & c), Inaba LPS–specific (Figure 4.5.11 d, e & f) and Ogawa LPS-specific (Figure 4.5.11 g, h & i) IgA antibody titres determined by end-point ELISA. (Figure 4.5.12 presents a timeline of CTB (Figure 4.5.12 a), Inaba-LPS (Figure 4.5.12 b) and Ogawa-LPS (Figure 4.5.12 c) specific IgA responses.

Mice receiving α-Galcer and CT as adjuvants had higher CTB-specific IgA titres in faecal pellet supernatants than mice receiving the antigens alone at all-time points (Figure 4.5.11 a-c). Significant differences between CT and α-Galcer were detected after 2 vaccinations but not after 3 (Figure 4.5.11 b). Although not significant, mice receiving CT instead of α-Galcer had slightly higher faecal pellet CTB-specific IgA titres after 3 rounds of vaccination. Over the course of the experiment, mice vaccinated with CT and the antigen mixture exhibited a significantly stronger induction of CTB-specific IgA titres compared to mice vaccinate with α-Galcer and the antigen mixture or the mixture alone (Figure 4.5.12 a). α-Galcer did however, significantly enhance the induction of CTB-specific IgA titres compared to antigens alone (Figure 4.5.12 a).

No significant differences in Inaba LPS-specific IgA titres where determined between different groups at any of the time points (Figure 4.5.11 d-f). However, mice vaccinated with antigen and α-Galcer consistently displayed higher titres than those vaccinated with antigen and CT (Figure 4.5.11 d-f). While a stronger induction of Inaba LPS-specific IgA was observed in mice vaccinated with α-Galcer compared to CT, this difference was not significant (Figure 4.5.12 b). However, compared to mice vaccinated with antigens alone,

only α-Galcer elicited a significant increase in IgA induction compared to mice vaccinated with antigen alone (Figure 4.5.12 b).

Interestingly, a significant difference between the ability of CT and α-Galcer to enhance faecal Ogawa LPS-specific IgA titres were observed after 2 and 3 rounds of vaccination (Figure 4.5.11 h and i). Furthermore, mice administered with α-Galcer and the vaccine mixture exhibited a significantly stronger induction of Ogawa LPS-specific IgA titres compared to both mice receiving the antigen mixture alone or together with CT (Figure 4.5.12 c). No significant differences were observed between responses in mice receiving antigens alone or in combination with CT in terms of Ogawa-LPS specific IgA titres (Figure 4.5.11 g-I, Figure 4.5.12 c).

4.5.11 – α-Galcer and CT differentially enhance tissue IgA responses to particulate and soluble antigens.

CT and α-Galcer both enhanced antigen-specific IgA induction in faecal pellet samples. However, CT more potently enhanced anti-CTB IgA (Figure 4.5.11 a-c and 4.5.12 a), while α-Galcer elicited stronger responses against both Inaba and Ogawa-LPS (Figure 4.5.11 d-i and 4.5.12 a and b).

To determine if these effects reflected differential antibody titres in intestinal tissue, mice were vaccinated as described in 4.5.10. On day 42 after the first vaccination, mice were sacrificed by cervical dislocation and perfused with heparin PBS. 3cm of the small intestines were dissected out and treated with saponin to extract the inter-tissue antibodies. CTB-specific (Figure 4.5.13 a), Inaba LPS–specific (Figure 4.5.13 b) and Ogawa LPS-specific (Figure 4.5.13 c) IgA antibody titres in the supernatants were determined by end-point ELISA.

CTB-specific IgA titres in the upper small intestines were significantly elevated in mice receiving either CT or α-Galcer together with antigen alone over those mice receiving just the antigen (Figure 4.5.13 a). Consistent with observations in faecal pellet supernatants (Figure 4.5.11 b), significant differences were found between the enhanced titres in mice receiving CT or α-Galcer together with antigens (Figure 4.5.13 a). While no significant differences were found between IgA titres against Inaba or Ogawa LPS in mice vaccinated with CT or α-Galcer together with antigens, mice receiving α-Galcer displayed slightly elevated titres (Figure 4.5.13 b and c), which may correlate with the findings in the faecal pellet supernatants (Figure 4.5.11 d-i).

4.5.12 – CT and α-Galcer differentially enhance IgA responses to particulate and soluble antigens in the serum.

In order to determine if the observation that CT and α-Galcer were optimal adjuvants for CTB and WCK bacteria respectively was restricted to the local mucosa or if this would also be observed in the systemic immune system serum antibody responses were assessed. Following vaccination as described in 4.5.10, serum was recovered following tail vein collection and centrifugation on day 41 and CTB-specific and Inaba LPS–specific IgA (Figure 4.5.14 a and c) and IgG1 (Figure 4.5.14 b and d) antibody titres were determined by end-point ELISA.

CTB-specific IgA and IgG1 titres were found to be significantly elevated in mice vaccinated with CT together with antigens compared to mice receiving α-Galcer (Figure 4.5.14 a and b). Interestingly, the opposite was observed when Inaba-LPS specific IgA was measure in the serum. Mice receiving α-Galcer instead of CT with antigens elicited significantly stronger antigen-specific IgA titres than mice receiving CT (Figure 4.5.14 c). No significant differences were found between any groups when serum Inaba-LPS specific IgG1 titres were measured (Figure 4.5.14 d).

4.5.13 – Co-administration of CT together with *V. cholerae* antigens enhances both local and systemic IL-17 producing cells.

Th17 cells and their cytokines have been implicated in the vaccine mediated immune response to enteric infections (discussed in detail in chapter 5). Peptide CTB (pCTB) was recently identified as a possible T cell receptor (TCR) epitope against CTB, which in its native form displays a strong immunomodulatory potential, often skewing results *in vitro* preventing its use as a T cell antigen for re-stimulation *ex vivo* [342].

To compare the ability of CT and α-Galcer to drive Th17-like responses *ex vivo* following re-stimulation after oral vaccination (detailed in 4.5.10), on day 42 after primary vaccination spleens and mesenteric lymph nodes (MLNs) were recovered, cells isolated and restimulated *ex vivo* with T cell medium, pCTB Low (10μg/ml), pCTB High (50μg/ml), Hikojima Bacteria (FK MS1346 *V.cholerae* (1 bacteria/cell)) or anti-CD3 (0.5μg/ml) in combination with PMA (25ng/ml). After 72 hours, supernatants were collected and analysed for the cytokine IL-17 by ELISA.

Splenocytes and MLN lymphocytes isolated from mice immunised with antigens and CT exhibited significantly stronger IL-17A production after re-stimulation compared to mice receiving antigens alone or together with α-Galcer (Figure 4.5.15). No significant differences were detected in IL-17A production between splenocytes or MLN cells from mice vaccinated with antigen alone or together with α-Galcer (Figure 4.5.15). No responses to pCTB were detected in any of the groups (Figure 4.5.15).

4.5.14 – The CTB-specific immunity elicited by CT leads to robust protection against toxin challenge.

Both CT and α-Galcer significantly enhanced the faecal anti-CTB IgA response following oral vaccination (Figure 4.5.11 c).

In order to compare the protective efficacy of these formulations following oral vaccination (see 4.5.10), on day 42, groups of mice were orally challenged with CT (20μg per mouse). After 6 hours mice were sacrificed by cervical dislocation. The GIT was dissected out, the intestine was photographed (Figure 4.5.16 a) and caeca isolated and weighed. Caeca were dried for 7 days at 37°C and weighed again to calculate the total percentage water lost to evaporation (Figure 4.5.16 b).

Only mice receiving antigen together with CT were fully protected from OCTC, qualified as a non-significant difference in percentage caecum fluid compared to un-challenged (healthy) mice (Figure 4.5.16 b) and an absence of fluid accumulation in the GIT (Figure 4.5.16 a). In contrast mice receiving α-Galcer and antigens were not fully protected from OCTC (Figure 4.5.16). However, these mice had a significantly lower percentage of fluid accumulation in the caecum compared to mice receiving antigens alone (Figure 4.5.16 b).

4.5.15 – A novel whole cell killed strain of *V.cholerae* supplemented with CTB and adjuvanted with α-Galcer delivered in SmPill elicits stronger antigen-specific faecal pellet titres than Dukoral®.

Dukoral® is currently the most widely used OCV. However, it is limited by its high price and relatively low immunogenicity. FK.MS1346 *V.cholerae* supplemented with CTB α-Galcer enhanced faecal antibody titres similarly to CT (Figure 4.5.11 and 4.5.12).

In order to determine if SmPills loaded with FK.MS1346 *V.cholerae* and CTB together with α-Galcer could elicit comparable immune responses to Dukoral®, mice were orally as per figure 4.5.1 with PBS as a control. Treatment groups included mice vaccinated with Dukoral® (27.5μg CTB and $3x10^8$ Bacteria per mouse) in solution or with SmPills containing CTB (27.5μg per mouse) and MS1346 *V.cholerae* ($3x10^8$ Bacteria per mouse) with or without α-Galcer (10μg) as an adjuvant. Faecal pellets were collected on days 13, 27, 41 and CTB (Figure 4.5.17 a, b, & c), Inaba LPS (Figure 4.5.17 d, e & f) and Ogawa LPS-specific (Figure 4.5.17 g, h & i) IgA antibody titres determined by end-point ELISA. A timeline summarizing the induction of anti-CTB (Figure 4.5.18 a), Inaba-LPS (Figure 4.5.18 b) and Ogawa-LPS (Figure 4.5.18 c) IgA responses is also presented.

Oral vaccination with SmPills containing FK.MS1346 *V.cholerae* and CTB together with α-Galcer elicited significantly higher anti-CTB faecal IgA titres compared to Dukoral® after 3 rounds of oral vaccination (Figure 4.5.17 c). No significant differences were detected between mice receiving SmPills with FK.MS1346 *V.cholerae* and CTB alone and Dukoral® (Figure 4.5.17 c). Analysis of the samples over the entire time course of the study revealed that the addition of α-Galcer to SmPills containing FK.MS1346 *V.cholerae* and CTB significantly enhanced the induction of faecal CTB-specific IgA titres over SmPills with antigen alone but the response was not significantly greater than the Dukoral® group (Figure 4.5.18 a).

Oral vaccination with SmPills containing FK.MS1346 *V.cholerae* and CTB together with α-Galcer resulted in enhanced faecal anti-Inaba LPS titres and their induction compared to non-adjuvanted SmPill formulation or Dukoral® after 2 and 3 rounds of vaccination (Figure 4.5.17 e and f, .figure 4.5.18 b). Inclusion of α-Galcer as an adjuvant also led to a significantly enhanced Ogawa LPS-specific IgA titres compared with SmPills containing either antigen alone (Figure 4.5.17 I, (Figure 4.5.19 c) but not when compared with Dukoral®.

4.5.16 – Dukoral® and SmPills containing antigen and α-Galcer elicit similar antigen-specific intestinal tissue IgA titres.

To determine the ability of a candidate OCV delivered in SmPills and Dukoral® to elicit local intestinal tissue IgA responses, following vaccination (see 4.5.15) mice (day 42) were sacrificed by cervical dislocation and perfused with heparin PBS. 3 cm of the small intestines were dissected out and treated with saponin to extract the inter-tissue antibodies. CTB-specific (Figure 4.5.19 a), Inaba LPS–specific (Figure 4.5.19 b) and Ogawa LPS-specific (Figure 4.5.19 c) IgA antibody titres were determined by end-point ELISA.

No significant differences in CTB, Inaba LPS or Ogawa LPS-specific IgA titres were detected in the intestines of mice vaccinated with SmPills containing FK.MS1346 *V.cholerae* and CTB together with α-Galcer or Dukoral® (Figure 4.5.19 a, b and c). Significantly lower CTB-specific IgA titres were detected in mice that received SmPills containing antigen alone than in those that received SmPills containing α-Galcer (Figure 4.5.19 a).

4.5.17 – The serum antibody titres generated after oral vaccination with either Dukoral® or SmPills containing antigens and α-Galcer are similar.

No significant differences in serum (collected day 42) CTB or Inaba LPS-specific IgA or IgG1 titres were detected in the serum of mice vaccinated (as per 4.5.15) with Dukoral® or SmPills containing FK.MS1346 *V.cholerae,* CTB and α-Galcer (Figure 4.5.20). However, vaccination with SmPills containing α-Galcer elicited significantly higher serum IgA titres against CTB and Inaba LPS than SmPills containing antigen alone (Figure 4.5.20 a and c).

4.5.18 – Neither Dukoral® nor SmPills containing antigen and α-Galcer protects against oral cholera toxin challenge.

Dukoral® has been shown to provide short-term protection from cholera toxin due to the incorporation of CTB into the formulation. In a previous study, a formulation composed of α-Galcer together with FK.MS1346 *V.cholerae* and CTB delivered in solution did not elicit strong protection from oral cholera toxin challenge (Figure 4.5.16).

In order to determine if incorporation into SmPills can enhance to ability of an OCV candidate composed of α-Galcer together with FK.MS1346 *V.cholerae* and CTB to provide protection against OCTC, vaccinated mice (see 4.5.15) were orally challenged with CT (20μg per mouse). After 6 hours mice were sacrificed by cervical dislocation. The GIT was dissected out and the intestine photographed (Figure 4.5.21 a) and caeca isolated and weighed. Petri dishes containing the caecum were placed in an oven for 7 days at 37°C and weighed again to calculate the total percentage water lost to evaporation (Figure 4.5.21 b).

All vaccinated mice exhibited significantly higher fluid accumulation in their caeca compared to un-challenged (healthy) mice (Figure 4.5.21 b). Only one mouse in the Dukoral® vaccinated group had a caecum fluid content of less than 80% (Figure 4.5.21 b). Furthermore, as evident from the photographs there is considerable fluid accumulation in the GIT of most vaccinated mice, with the exception of 1 Dukoral® vaccinated mouse (Figure 4.5.21 a).

4.4 – Discussion

V. cholerae is a pathogenic bacterium that causes the diarrhoeal disease cholera. Cholera diarrhoea is characterised as a profuse watery diarrhoea often called "rice-water stool" due to the cloudy appearance of the diarrhoea (Figure 1.28). This bacterium causes significant morbidity and death in both endemic and epidemic regions [211]. Cholera is spread through food and water that becomes contaminated by the bacterium. The most common cause of the dissemination of this pathogen is inadequate sanitation and a lack of clean drinking water. Many of the endemic regions are developing countries, where clean water and sanitation are difficult to implement due to cost and the socio-political environment. Furthermore cholera can rapidly spread during natural disasters and war. However, when introduced into an un-infected region *V. cholerae* can rapidly disseminate and infect the local population leading to a full-scale epidemic. There is no better example of this phenomenon that Haiti [278]. In 2010 the Caribbean island of Haiti suffered a disastrous earthquake. In the aftermath of this catastrophe, the nation was almost entirely dependent on foreign aid. The United Nations responded with a large contingent of international troops to aid with the humanitarian effort. One detachment, belonging to the Nepalese military established its base of operations close to a river, upstream from a location where locals collected drinking water. The latrines for this camp were placed close to the river, and troops who were carrying *V. cholerae* unbeknownst to them contaminated the water. This lit the fuse of a bomb that exploded into one of the worst Cholera epidemics seen in many years. Cases on the island alone increased the global statistic by 194,000 cases and 3,819 deaths [278]. In order to stem epidemics and protect uninfected individuals the WHO has recommended that OCV programs be used during outbreaks for several years [343].

Although numerous OCVs have been developed and three are licenced for use in humans and display efficacious results in foreign travellers to endemic regions [212], these often generate only poor immune responses in local inhabitants. Furthermore, these vaccines all require cold-chain storage and transport to the medical centres where they are administered [344]. Current oral vaccines are manufactured as lyophilized powders which require re-constitution in clean water prior to use [212]. Dukoral® for instance comes as part of a kit containing the vaccine and a sachet of sodium hydrogen carbonate buffer. The kit requires the user to mix the sachet with 150ml of clean water then add the vaccine from a vial. Dosing must be performed 3 times over the course of 6 weeks. During this time, the un-reconstituted vaccine must be kept refrigerated [212]. During periods of disaster and conflict, refrigeration and the utilities required to sustain this are often damaged or absent. Furthermore, transport into these regions may be restricted to air, which has limit scope for refrigeration. Enhancing the immunogenicity of oral vaccines has been seen as key to improving their efficacy [285]. WCK OCVs are currently the only licenced cholera vaccines for human use.

By examining protective antigens in vaccines such as Dukoral® it is possible to determine the antigenic epitopes that responses are mounted against. The responses elicited by Dukoral® are intestinal SIgA against the O-antigen of LPS (either Inaba or Ogawa) and anti-toxic SIgA against CTB [285]. This vaccine, which is the most efficacious OCV thus confers both anti-bacterial and anti-toxic protection. The dual functionality of this vaccine is provided by a combination of three WCK Inaba or Ogawa expressing O1 *V. cholerae* bacteria and the subunit component rCTB [212]. In order to improve the Dukoral® formulation it is necessary to dissect the vaccine into its two components; the WCK bacteria and the subunit antigen. CTB is a non-toxic subunit of the CT holotoxin and has been shown to possess strong antigenic properties. When a memory SIgA response is established against CTB it prevents the CT holotoxin from binding to GM1 rendering it unable to initiate its ADP-ribosylating

effects and so blocking the pathogenesis of *V. cholerae* [285]. In contrast to neutralising antibodies against CTB, anti-LPS antibodies against the O-antigen of *V. cholerae* LPS lead to the clearance of the bacterium via increased solubility in the mucus.

The use of subunit antigens for oral vaccines comes with both advantages and challenges. Using subunit antigens is regarded as the safest method for establishing immune responses [12]. However, oral subunit vaccines are very susceptible to degradation by acids in the stomach and proteases in the duodenum [12]. Subunits, often composed of proteins are often poorly antigenic and so require the inclusion of adjuvants to boost their immune-stimulatory properties [12]. CTB on the other hand is regarded as a strong candidate antigen for an oral subunit vaccine against cholera [345]. CTB has also been included in a licenced vaccine and has been shown to be safe and well tolerated in humans, making it a versatile subunit antigen [285]. The research group led by Kiyono showed that CTB expressed in rice plants could be used to elicit strong SIgA titres in mice which protected mice from OCTC [339]. However, whether this prototype vaccine will elicit protection in humans remains to be demonstrated. Furthermore, negative public opinion regarding genetically modified crops may hamper uptake of such a prophylactic strategy.

While SmPills enhanced the ability of a novel oral ETEC vaccine; FK.ETEC to drive mucosal antibody responses (Figure 3.5.6 & 3.5.8), currently no murine model of ETEC infection exists to evaluate the protective efficacy conferred by these antibodies. While suckling pigs are naturally susceptible to ETEC and models of infection have been established, logistics and financial implications of this model make establishing it challenging [237]. In contrast to ETEC, several models of cholera, specifically cholera toxin induced diarrhoea have been established in rodents. The most commonly used rodent models of cholera include intestinal loops, intestinal ligation and OCTC (section 2.2.5). The mouse OCTC model is relatively simple to conduct and is both effective and accepted by the scientific community. While

different mouse strains require an adapted protocol, the readouts of this challenge remain identical. Firstly, the visual impact of susceptibility to OCTC in mice is striking with a large amount of fluid accumulating in the lumen of the small intestine and caecum together with the absence of solid faecal matter in either (Figure 2.1). Secondly, the percentage of fluid in the caecum can also be evaluated by weighing the "wet" caecum, drying it for a period of days and weighing the "dry" caecum (section 2.2.5.4). Expressing the difference in terms of the percentage water lost to the drying process allows for the quantification of the severity of the diarrhoea as a high percentage of fluid in the caecum is indicative of susceptibility to OCTC.

While muco-rice was shown to protect mice from OCTC [339], even high doses of CTB incorporated in SmPills failed to elicit sufficiently high intestinal IgA titres to protect mice from oral CT challenge (Figure 4.5.2). To ensure that accurate loading of SmPills with CTB was achieved in figure 4.5.2 and that errors in dosing did not account for the lack of protective efficacy from these SmPills, the required dose of SmPills were dissolved and supernatants run on a SDS-PAGE gel (Figure 4.5.3). In comparison to reference concentrations of CTB after dilution, no recognisable difference between the bands correlating to different concentrations of CTB in SmPills were found (Figure 4.5.3). Although this method is not quantitative, these findings suggest that accurate loading in SmPills is possible and that errors in this did not account for the poor efficacy of these formulations (Figure 4.5.2).

As a subunit antigen, CTB is more vulnerable to damage and destruction through manufacturing processes than WCK antigens such as FK.ETEC which have often been stabilised by inactivation via heat or formalin treatment and rendered more resistant to degradation by the virtue of being a whole cell bacterium [12]. Furthermore CT holotoxin, being heat-labile is also sensitive to harsh production techniques which can destroy the molecule. As CTB alone did not elicit protective immune responses (Figure 4.5.2) it was clear that an adjuvant needed to be included in the formulation. In order to determine the

impact of the SmPill manufacturing process (outlined in section 3.4) on a labile adjuvant it was decided to incorporate CT as an adjuvant as this is both the gold standard mucosal adjuvant and sensitive to harsh production methods which would allow the evaluation of both the production process and the mucosal efficacy of SmPills for subunit antigens and protein-based adjuvants.

No significant differences were found between faecal anti-CTB IgA titres in mice vaccinated with CTB and CT administered either in solution or in SmPills after 3 rounds of vaccination (Figure 4.5.4), while induction was reduced in SmPills (Figure 4.5.4 c), due to a significantly lower titres after 2 rounds of vaccination (Figure 4.5.4 a). For both delivery methods the co-formulation of CT with CTB significantly enhanced the antigen specific titres elicited (Figure 4.5.4). Serum IgA titres are often used as an indicator of an oral vaccine's efficacy in humans [265, 323]. Additionally, many injectable vaccines (such as those against tetanus and HPV) are composed of subunit antigens [116] and even though these vaccines are highly effective, their administration by the oral route could greatly improve the reach of vaccine programs and reduce the associated costs. Differences in serum antibody titres against CTB were not significantly different when delivered by SmPill or in solution when CTB was co-formulated with CT (Figure 4.5.5). The ability of a prototype OCV to provide protection against an infectious agent is the true test of a vaccines potential efficacy. Following OCTC, mice receiving either SmPills or a solution containing CT and CTB were fully protected from CT-induced fluid accumulation (Figure 4.5.5). This suggests that the process used to manufacture SmPills is sufficiently mild to allow for both the incorporation of protein based subunit antigens and heat-labile adjuvants such as CT or LT and their derivatives. However, as previously mentioned CT is too toxic for use in humans [346]. Although a dose-dependent decrease in caecum fluid content was observed at higher doses of CTB in SmPills (Figure 4.5.2 b), the formulation was not fully protective. This observation does suggest that perhaps

doses higher than 60μg of CTB in SmPills may result in protection although the high cost of producing recombinant CTB makes this an unfeasible option. Therefore the inclusion of efficacious and tolerated adjuvant may be required. The ability of α-Galcer to enhance the mucosal efficacy of FK.ETEC was demonstrated in chapter 3. Furthermore, McNeela *et al*, also showed that co-formulation of OVA with α-Galcer in SmPills enhanced the immune response against this subunit antigen (Figure 3.1.6 b).

These results strongly support the potential of SmPills to enhance the oral efficacy of CTB when co-formulated with CT. Vaccination against CTB has been shown to not only enhance the short-term protective efficacy of existing OCVs but also provide cross-protection against ETEC induced diarrhoea due to the high structural similarity between LTB and CTB [295]. Interestingly, a synergy was noted between the co-administration of LTB and WCK ETEC which resulted in higher antibody titres than when each component was administered alone [315]. While a CTB subunit vaccine will protect the vaccinee from the effects of CT during cholera infection, the bacteria that produce this agent may not necessarily be protected against. Furthermore, as shown by oral polio vaccine (OPV), oral vaccines and indeed vaccines in general act by not only protecting the individual from the infection but also through promoting herd immunity [347]. Herd immunity can protect unvaccinated individual through different mechanism. Many illnesses rely on infected individual to act as not only as a reservoir and site of reproduction for infectious agents but also as a transmission aid (e.g through the act of coughing and sneezing influenza is spread, thus contaminating surfaces and uninfected individuals allowing the spread of the illness by direct or indirect contact). However, by vaccinating individuals against a disease one not only protects that subject directly but one also removes the ability of that person to act as a reservoir and transmission aid for the virus and therefore, by doing so it protects un-vaccinated individuals from contracting the infection.

However, in the case of oral vaccines, such as OPV and OCVs these can act by similar and yet distinct means, primarily as the transmission route and disease characteristics are different. In the case of polio and *V. cholerae* the reservoirs of infection are often bodies of water. Both diseases are spread through the consumption of this contaminated water and food, or contact with infected faeces. When these illnesses infect people they spread via faeces, albeit by different mechanisms. Cholera, by the virtue of being a diarrhoeal disease produces large volumes of "rice stool" which contain active vibrios. Polio on the other hand does not induce diarrhoea, but instead the virus sheds from IECs and can be found on faecal samples from infected individuals. Recent insights into herd immunity in Israel suggest that although the injectable polio vaccine (IPV) confers high levels of personal protection, the low mucosal immunogenicity of this vaccine has led to the re-emergence of polio in an area that was thought to have been cleared of infectious agent [347]. It is known that the OPV halts the transmission of polio virus, while the IPV does not. Therefore IPV polio programs may need an OPV element to not protect an individual, but prevent reservoirs from replenishing over time[347]. By using faeces as a transmission route both diseases can directly contribute to the infectious burden in an endemic or out-break region (such as Haiti and North Syria for cholera and polio respectively) by supplementing the reservoir of infectious agent in a region, thus allowing for the infection of more individuals and a further contribution of new bacteria and viruses to the pool [347]. Environmental analysis of regions with polio vaccination programs has detected a reduction in "wild" polio virions after implementation of such a program. This offers the possibility of effectively "cleansing" an endemic region of the disease-causing agent by removing a key step in the pathogenic cycle namely reproduction and transmission.

When developing a cholera vaccine that can be implemented in a large scale public health effort such as those in endemic locations or during rapid response to an outbreak one must

consider several factors some of which have been already discussed such as ease or administration, storage and transport [10, 348].

In terms of an oral vaccine against cholera SmPills have not only been shown capable of being accurately dosed (Figure 4.5.3), providing protective immunity from OCTC (Figure 4.5.6) and potentially stable non-refrigerated temperature (Rosa, M. *personal communication*) but also capable of delivering a WCK antigen (section 3). To investigate the ability of SmPills together with α-Galcer to enhance the oral efficacy of a WCK vaccine based on the detoxified *V.cholerae* strain JS1569 which lacks the gene encoding CTA (*ctax*). However, unlike FK.ETEC which expresses a peptide antigen (CFA/I), JS1569 *V.cholerae* express Inaba LPS antigen. $3x10^8$ bacteria provided a sufficient dose to induce a strong response to CFA/I when mice were vaccinated with FK.ETEC (chapter 3). The dose used to manufacture the JS1569 SmPills was $2.5x10^8$ bacteria. The current method employed in the manufacture of SmPills to determine the loading of its components compares the ratio of weights of the various components of the emulsion including solutol, bacteria and α-Galcer together with the weight of gelatine and water added. After formulation of the un-coated beads they are weighed again after drying and again after coating. The individual beads are then weighed again to determine the percentage of enteric coat applied and the weight of the core containing the vaccine components. Using the initial weight of the individual components and the weight ratios of these in comparison to each other it is possible to determine the loading of each component in the SmPill. CTB can be accurately loaded into SmPills where the quantities estimated corresponded to those of reference amounts of CTB on an SDS-PAGE gel compared to the dissolved SmPills (Figure 4.5.3). In order to ensure accurate and intact loading of WCK bacteria into SmPills prior to use was possible, it was decided to develop an assay to serve as an important quality control mechanism similar to the method of determining CTB incorporation (Figure 4.5.3).

Protocols developed by Tobias *et al* in the research group of our collaborators in Gotenburg, Sweden have been successful at estimating the loading of intact WCK bacteria into SmPills using a *H. pylori* strain (*unpublished data*). Following on from this study, a method for the characterisation of bacterial numbers and LPS integrity after bacterial incorporation into SmPills was developed by Karlsson *et al* (Figure 4.5.7). This method was used to show the intact incorporation of JS1569 *V.cholerae* into SmPills (Figure 4.5.7)

When mice were orally vaccinated with WCK JS1569 bacteria co-administered with α-Galcer, mucosal IgA responses were enhanced over mice receiving WCK JS1569 alone (Figure 4.5.8). This effect was further enhanced when the formulation was encapsulated in SmPills (Figure 4.5.8). However, no enhanced faecal IgA response was elicited in mice receiving SmPills with JS1569 lacking α-Galcer (Figure 4.5.8). Furthermore, local intestinal tissue IgA responses against Inaba-LPS were enhanced when JS1569 *V. cholera* were co-formulated together with α-Galcer in SmPills (4.5.9). In all cases JS1569 bacteria did not elicit anti-Inaba LPS antibody responses in the absence of α-Galcer in SmPills (Figure 4.5.8 & 4.5.9). This observation is consistent with the previous observations that α-Galcer is required for the optimal function of SmPills to elicit robust mucosal and systemic immunity (Figure 3.5.11 & 3.5.15).

One striking observation in this study was that although in all readouts of the immune response to the oral vaccine high responses were detected in responding mice, the number of mice responding to the vaccine was not optimal (~60% for mice receiving the formulation of JS1569 *V.cholerae* together with α-Galcer in SmPills). Furthermore, no significant benefit was observed when α-Galcer was added to JS1569 bacteria in solution or if the formulation was encapsulated in SmPills on anti-LPS serum IgA and IgG1 antibody levels (Figure 4.5.10). Although the accepted threshold of herd immunity is approximately 80-85% for most injectable vaccines it has yet to decisively confirmed for cholera. Simulations suggest that in

some endemic regions a coverage rate of 50% may be sufficient to protect non-vaccinated individuals, provided significant naturally acquired background immunity to the disease exists in the population. In non-endemic areas of outbreak with naïve populations it is likely that in excess of 70% of the population needs vaccination to provide adequate herd immunity with this figure being significantly influenced by the presence of clean drinking water and sanitation [349, 350]. Therefore it will be most likely necessary to increase the dose of WCK *V.cholerae* in subsequent studies to ensure sufficient antigen is available to elicit an immune response.

One strategy for improving the immunogenicity of an oral vaccine is to increase the antigen load that is delivered per dose. However this does not necessarily mean the arbitrary increasing of the concentration of antigen in a vaccine (e.g. a higher dose of bacteria) but rather increasing the expression of antigen on each individual bacterium [307]. Another significant obstacle to the more widespread use of OCVs is the costs associated with production. All three currently available WCK OCVs require the production of different batches of various *V. cholerae* strains which must then be inactivated by different mechanisms to ensure that the heterogeneous mix of antigenic targets are all incorporated in the final vaccine mixture [285]. While both Inaba and Ogawa serotypes have distinct LPS molecules [287], a naturally occurring Hikojima O1 serotype *V. cholerae* strain expresses both Inaba and Ogawa LPS [305, 306]. It is thought that the Hikojima strain is a transient state that occurs as the bacterium is switching from the Ogawa to the Inaba serotype. This mechanism aroused much interest as the generation of a stable Hikojima strain could significantly reduce the cost associated with the production of OCVs. This led to the group of Lebens *et al* generating a stable Inaba and Ogawa expressing O1 *V. cholerae* strain which was evaluated as a novel WCK OCV candidate [307]. This study showed that formalin killed Hikojima O1 *V. cholerae* elicited comparable anti-Inaba and Ogawa LPS antibody results to

Dukoral® after oral immunisation, with the obvious advantage being the need for only one batch of bacteria to be produced rather than the 3 batches for Dukoral® while still expressing Inaba, Ogawa and O1 antigenic targets [307]. Furthermore, this strain expressed high levels of Inaba (75-80%) and Ogawa (20-25%) LPS [307]. This strain is referred to as MS1346 *V.cholerae*.

At this point it was determined that the best vaccine formulation to put forward as a candidate OCV was the combination of FK.MS1346 *V.cholerae* and CTB together with α-Galcer. To compare the efficacy of this formulation to CT in terms of antibody production faecal pellet IgA titres against CTB, Inaba and Ogawa LPS were measured (Figure 4.5.11). α-Galcer elicited comparable CTB titres (albeit with a significantly slower induction (Figure 4.5.12 a)) to CT (Figure 4.5.11 a-c). However, very interestingly α-Galcer elicited slightly higher anti-Inaba LPS IgA and significantly higher anti-Ogawa LPS IgA titres (Figure 4.5.11 f and i respectively). A significantly faster induction of Ogawa LPS-specific IgA antibodies was also observed with α-Galcer compared to CT (Figure 4.5.12 c). This observation indicates that, whereas CT is an excellent adjuvant for soluble antigens, it may not be optimal for particulate antigens such as WCK bacteria. This is further supported by results from Abautret-Daly, where CT was found to be a less effective driver of faecal IgA responses against killed *H. pylori* than α-Galcer (unpublished data). This trend was also observed in intestinal tissue IgA (Figure 4.5.13) and serum IgA (Figure 4.5.14). This interesting observation may point towards a differential adjuvanticity between the two molecules which is dependent on the physical nature of the antigen. However, it may also be possible that the CT holotoxin used as an adjuvant may be benefitting from additional amounts of antigen due to its CTB subunit. In order to account for this variation of antigen concentration it may be necessary to calculate antigen amounts in molar concentrations and adjust the amount of CTB administered with α-Galcer to reflect that being additionally delivered with CT.

An important measure of an anti-toxic OCV is its ability to protect against OCTC. However, mice vaccinated with the bivalent FK.MS1346 *V.cholerae* and CTB formulation together with α-Galcer were not fully protected against OCTC in contrast to those vaccinated with CT (Figure 4.5.16). Although the mice vaccinated with FK.MS1346 *V.*cholerae, CTB and α-Galcer had significantly lower fluid accumulation in the caecum compared to those vaccinated with antigen alone, these still had significantly higher fluid percentages in the their caeca compared to healthy mice (Figure 4.5.16 graph). Interestingly, when T cell responses, specifically Th17-like responses were measured, cells from mice vaccinated with FK.MS1346 *V.cholerae* and CTB together with α-Galcer had significantly lower IL-17A production compared to mice vaccinated with CT (Figure 4.5.15). This was most strikingly observed in the MLNs, where α-Galcer vaccinated mice had no enhanced antigen-specific IL-17A production (Figure 4.5.15 b).

While α-Galcer potently enhanced both CTB-specific and LPS-specific IgA responses in a number of mucosal readouts, it did not sufficiently protect against OCTC. Encapsulation in SmPills could potentially enhance of efficacy of this formulation to provide protection against OCTC. It was decided to proceed and evaluate the ability of SmPills containing FK.MS1346 *V.cholerae* with CTB and α-Galcer to enhance mucosal IgA responses and to provide protection against OCTC in comparison to the clinically licenced vaccine Dukoral®.

The SmPill containing FK.MS1346 *V.cholerae* with CTB and α-Galcer elicited significantly enhanced anti-CTB and Inaba LPS IgA responses compared to Dukoral® in faecal pellet supernatants (Figure 4.5.17). The induction of Inaba LPS-specific faecal IgA responses was also significantly higher in SmPills compared to Dukoral® (Figure 4.5.18 a). Aside from inducing enhanced IgA titres compared to Dukoral®, SmPills also achieved these more rapidly.

Disappointingly however, neither Dukoral® nor SmPills containing FK.MS1346 *V.cholerae* with CTB and α-Galcer provided protection from OCTC (Figure 4.5.21). The reduced protection seen in figure 4.5.16 may therefore not be a result of the destruction of the vaccine in the stomach, but rather another formulation-specific problem. As mentioned previously, a comparison with the same molar dose of CTB needs to be performed to compare α-Galcer accurately to CT. However it may also be that α-Galcer is simply not the optimal adjuvant for use in an anti-toxic OCV. A mechanism of anti-toxin immunity beyond SIgA is proposed in chapter 5, which α-Galcer may not activate as efficiently as CT. In such a case it may be necessary to incorporate additional enterotoxin based adjuvants into the final formulation to engage other protective immune mechanisms. Furthermore, in both studies it was also noticed that the number of mice responding to the LPS antigens was approximately 50% so it may be necessary to further increase the dose of FK.MS1346 *V.cholerae* to achieve a higher response rate.

Despite the disappointing response of the candidate SmPill OCV to OCTC, it is clear from these results that α-Galcer is a potent driver of IgA responses against WCK LPS-based antigens and to some extent against subunit antigens. SmPills are also a viable candidate for the delivery of heat-labile adjuvants and protein adjuvants. While further optimization will be necessary to generate a candidate SmPill based OCV, it is clear from the data outlined in this chapter that the possibility of doing so may be more than a pipe dream, albeit maybe not as "SmPill" as had initially hoped.

4.5 – Figures

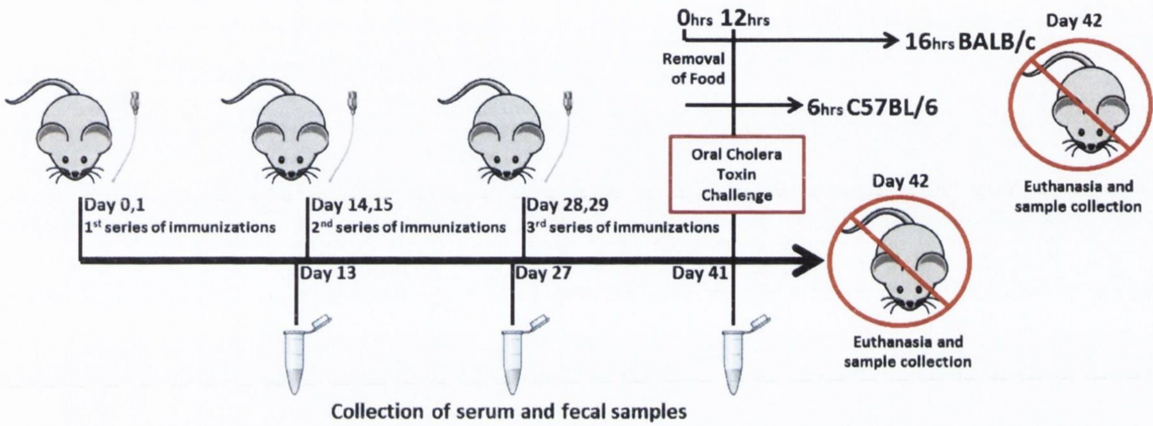


Figure 4.5.1 – Experimental Timeline for evaluating oral cholera vaccines. Mice were orally vaccinated on 2 consecutive days, for 3 rounds two weeks apart as shown above. Components of each vaccine are detailed in the respective figure legends for each experiment. Fresh faecal pellets were collected together with serum from tail bleeds on days 13, 27 and 41 for LPS-specific and/or CTB-specific antibody analysis performed on processed samples. On day 42 mice were sacrificed by cervical dislocation and organs isolated and samples harvested. In experiments where oral cholera toxin challenges were performed mice were starved for 12 hours on day 41 prior to challenge. If BALB/c mice were used mice were challenged 16 hours prior to cervical dislocation while C57BL/6 mice were challenged 6 hours prior to cervical dislocation on day 42.

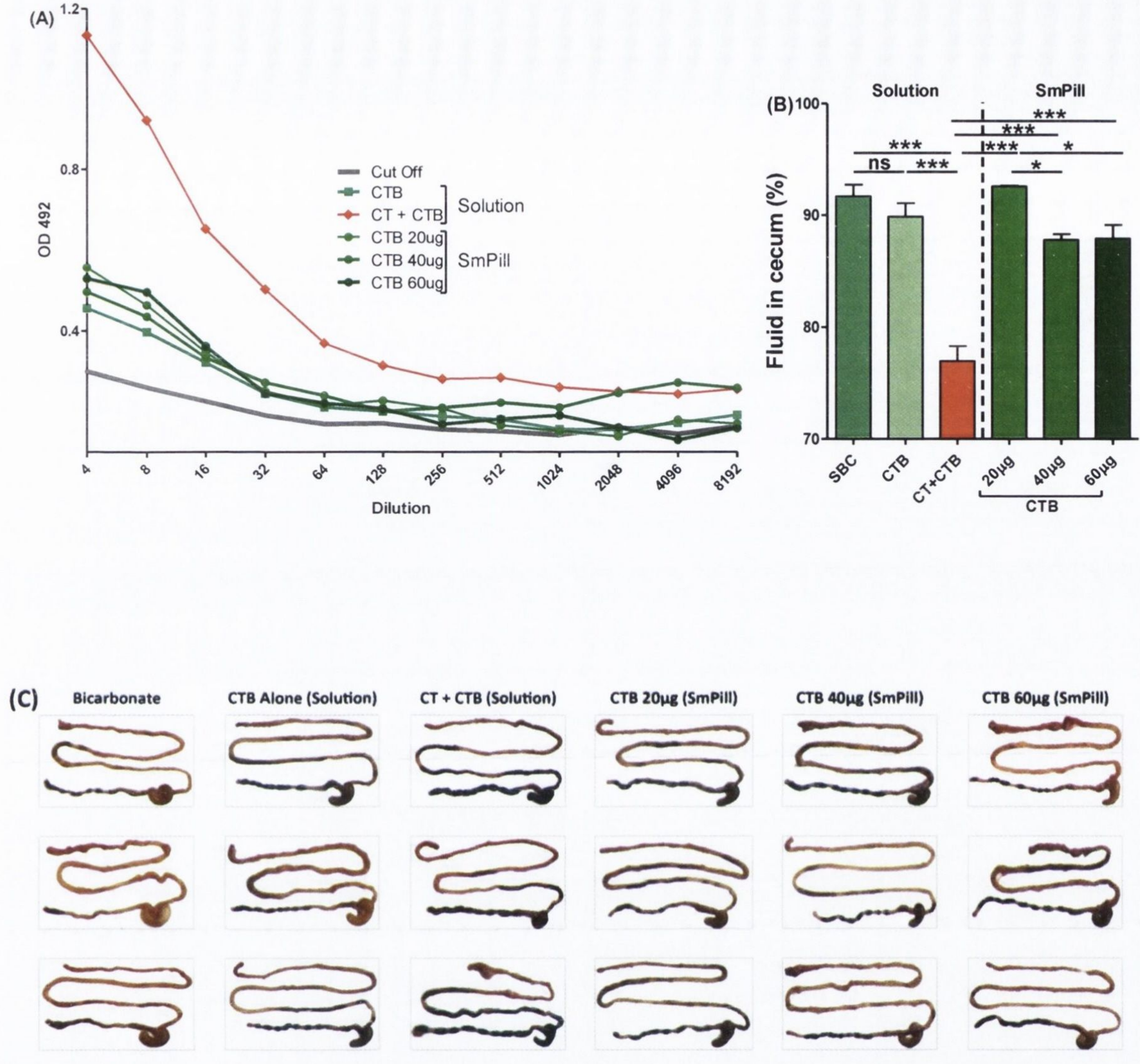


Figure 4.5.2 – High doses of CTB in SmPill formulations to not elicit protection against oral cholera challenge. BALB/c mice were immunised orally as per figure 4.5.1 with SBC as a control or CTB (20µg per mouse) either alone or mixed with CT (10µg) in solution. SmPills containing 20µg, 40µg or 60µg CTB were also used to vaccinate mice on those days. Fresh faecal pellets were collected and supernatants recovered following centrifugation on day 41 and CTB-specific IgA antibody titres in the supernatant were determined by end-point ELISA (a). On day 41, mice were orally challenged with CT (20µg per mouse). After 16 hours, mice were sacrificed by CO_2 asphyxiation. The GIT was dissected out, the caecum separated and weighed. Petri dishes containing the caecum were placed in an oven for 7 days at 37°C and weighed again to calculate the total percentage water lost to evaporation (b). Panel a presents mean OD492 values for all 5 mice per experimental group. Panel b presents mean % fluid in caecum (+ SEM) for all 5 mice per experimental group. Photographs of intestines were used to determine the presence or absence of diarrhoea (c). SBC versus CTB versus CT+CTB versus 20µg CTB (SmPill) versus 40µg CTB (SmPill) versus 60µg CTB (SmPill), * $p<0.05$, *** $p<0.001$, ns, not significant

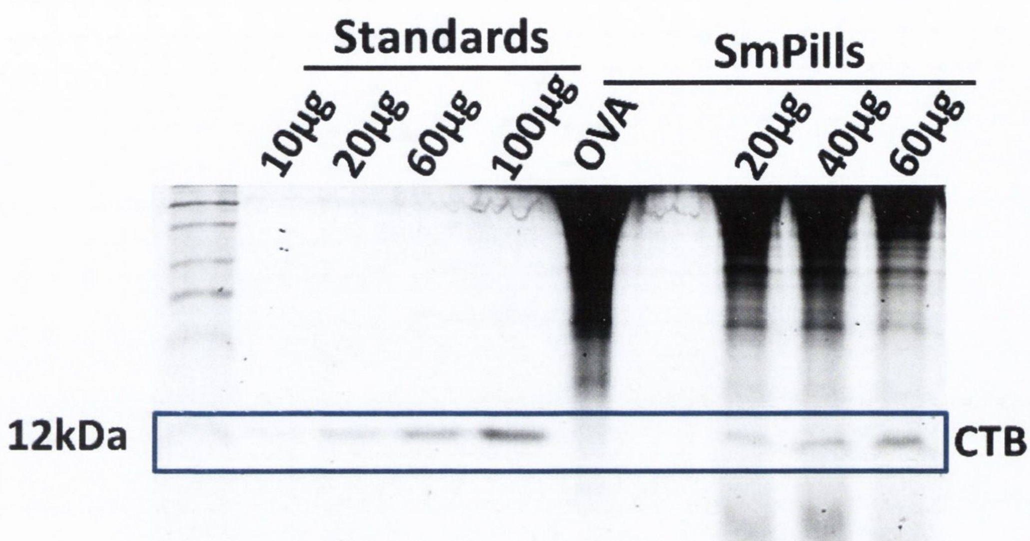
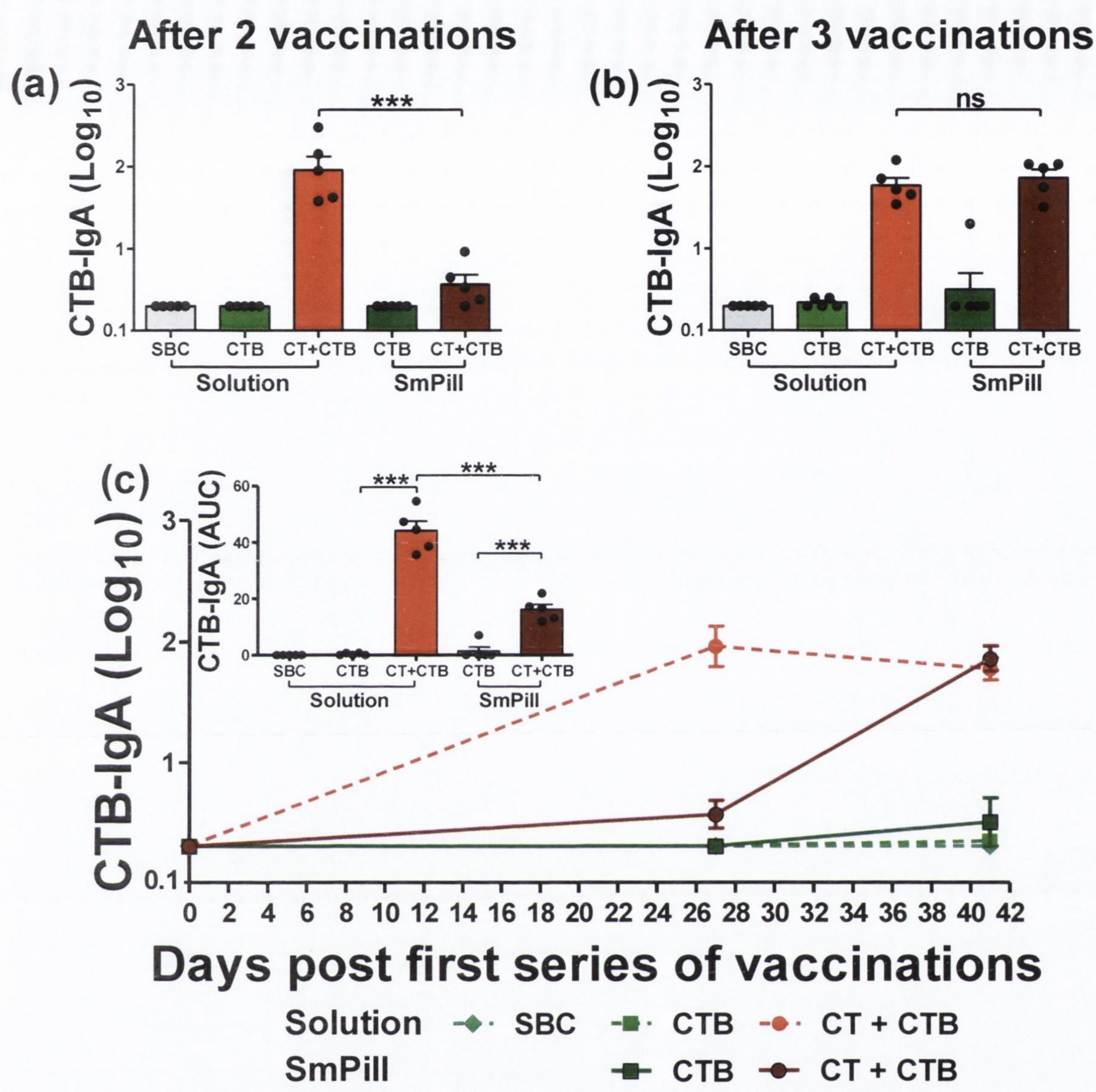


Figure 4.5.3 – Current SmPill manufacturing techniques ensure accurate loading of CTB. SmPills were dissolved in PBS at 50°C for 1 hour. Sample buffer was added to the tubes containing the SmPill supernatants and to CTB standards of known concentration. These tubes were then vortexed and heated for 3 minutes at 100°C. Samples were added to a 15% SDS-polyacrylamide gel and separated. Gels were placed in Coomassie blue stain overnight at room temperature. Several washes in destain buffer were performed until gels were scanned. Concentrations indicated above are representative of the initial amounts of CTB present in samples prior to dilution for running on the gel. Bands corresponding to CTB can be found at 12kDa.



Figures 4.5.4 – Techniques used in the production of SmPills do not attenuate the ability of a successful subunit vaccine formulation to elicit faecal IgA titres. BALB/c mice were immunised orally as per figure 4.5.1 with SBC as a control or CTB (20μg per mouse) either with or without CT (10μg) as an adjuvant with both formulations delivered either in solution or encapsulated in SmPills. Faecal pellets were collected on days 27 and 41. CTB-specific IgA antibody titres were determined by end-point ELISA. Panels a and b present mean titres (+ SEM) for 5 mice per experimental group. SBC (Solution) versus CTB (Solution) versus CT+CTB (Solution) versus CTB (SmPill) versus CT+CTB (SmPill), *** $p<0.001$, ns, not significant. Panel c presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. SBC (Solution) versus CTB (Solution) versus CT+CTB (Solution) versus CTB (SmPill) versus CT+CTB (SmPill), *** $p<0.001$.

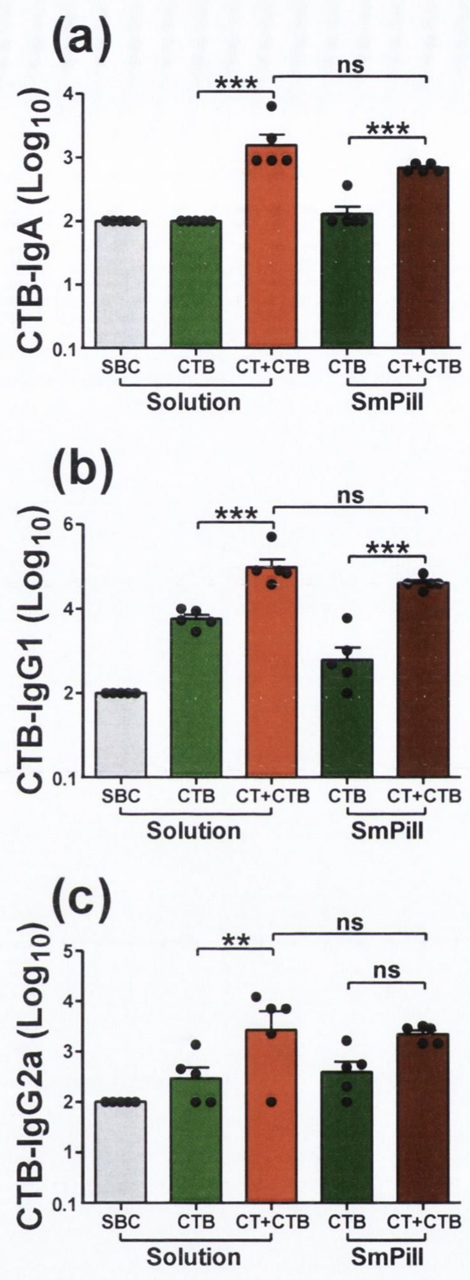


Figure 4.5.5 – Encapsulation in SmPills does not interfere with the ability of CTB and CT to elicit serum antibody responses following oral vaccination. BALB/c mice were immunised orally as per figure 4.5.1 with SBC as a control or CTB (20μg per mouse) either with or without CT (10μg) as an adjuvant with both formulations delivered either in solution or encapsulated in SmPills. On day 41 serum was obtained and CTB-specific IgA (a), IgG1 (b) and IgG2a (c) antibody titres determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. SBC (Solution) versus CTB (Solution) versus CT+CTB (Solution) versus CTB (SmPill) versus CT+CTB (SmPill), ** $p<0.01$. *** $p<0.001$, ns, not significant.

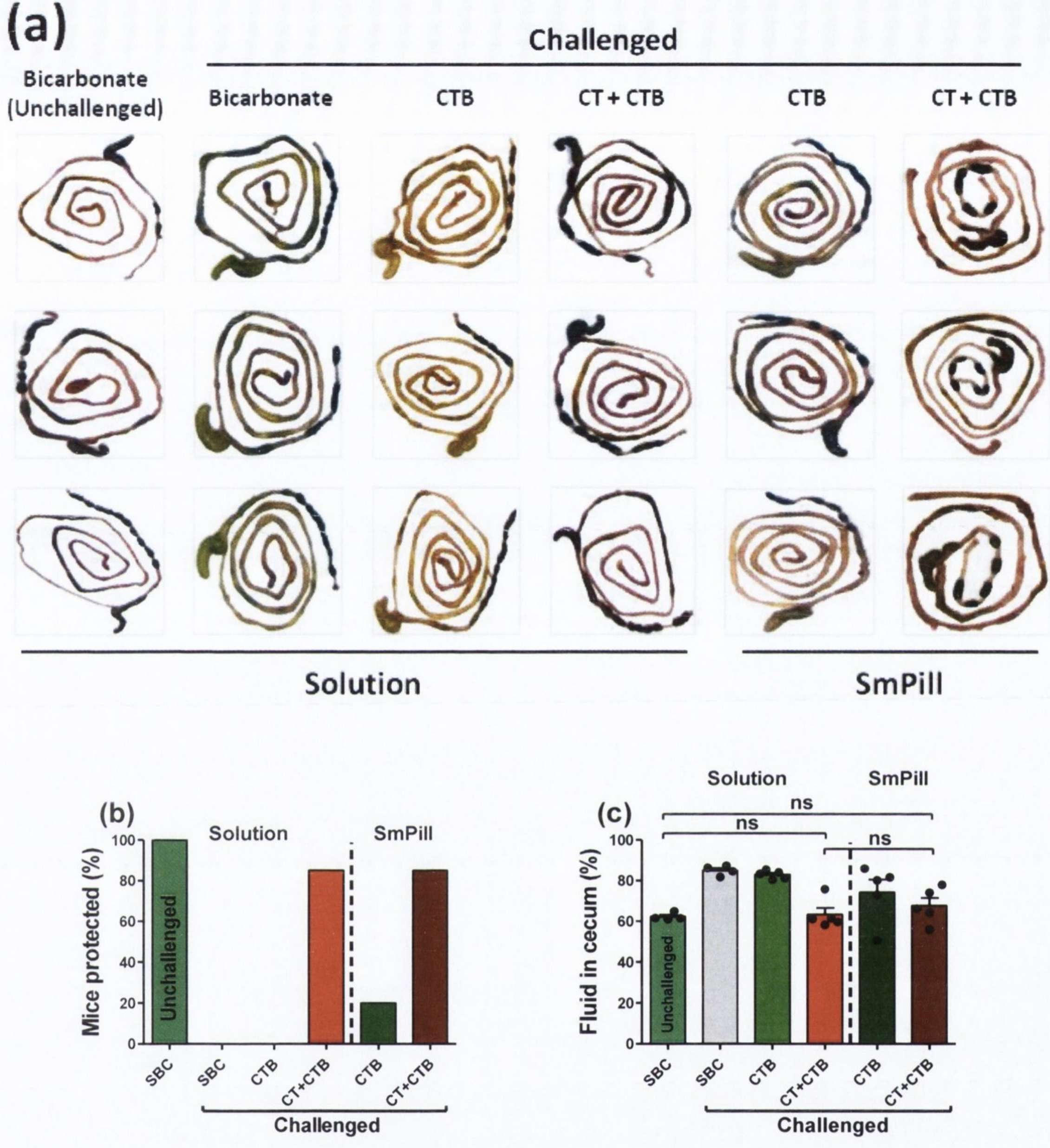
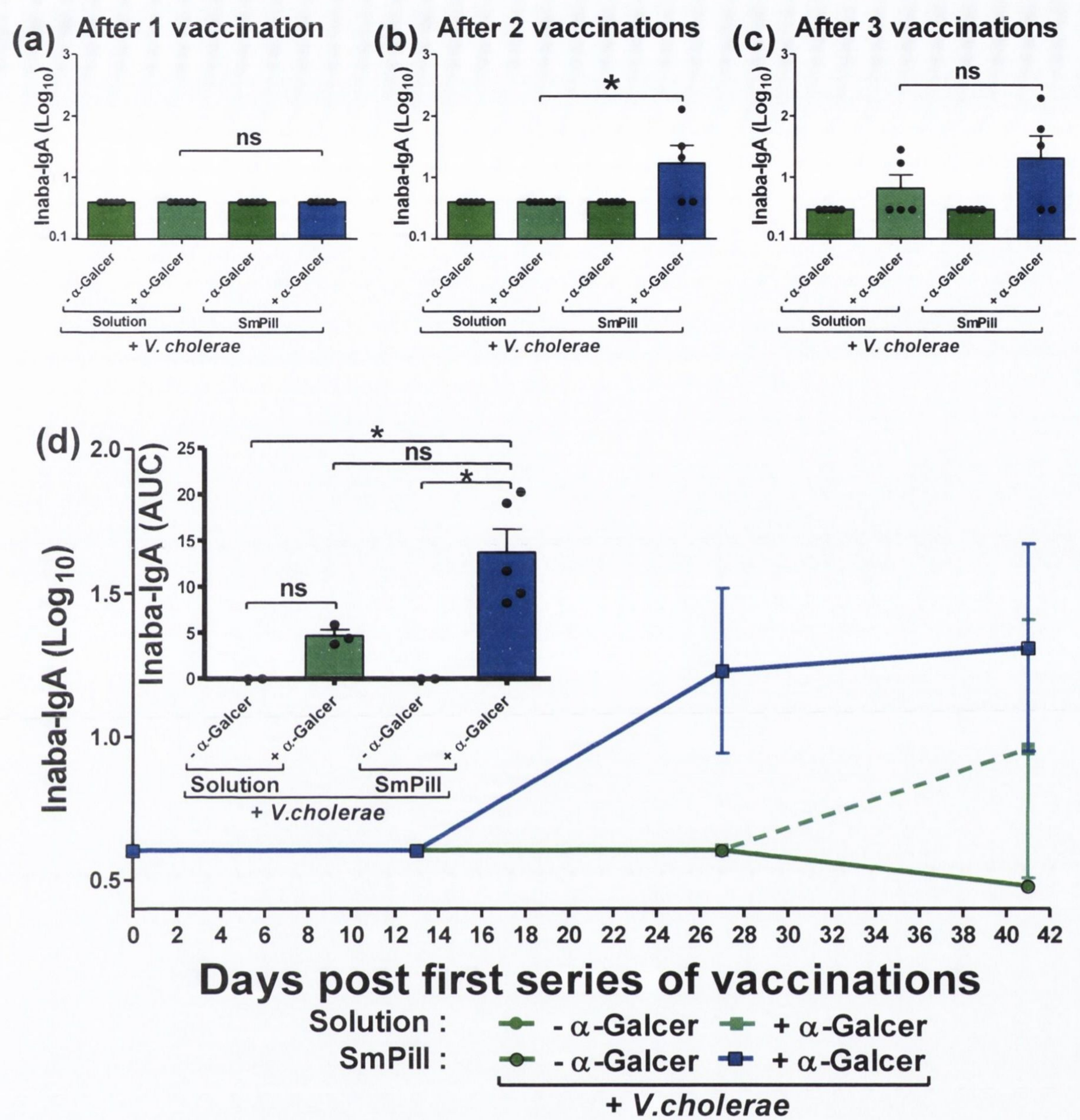


Figure 4.5.6 – CT and CTB does not lose its protective efficacy when administered in SmPills. BALB/c mice were immunised orally as per figure 4.5.1 with SBC as a control or CTB (20μg per mouse) either with or without CT (10μg) as an adjuvant with both formulations delivered either in solution or encapsulated in SmPills. On day 42, mice were orally challenged with CT (20μg per mouse). After 16 hours mice were sacrificed by CO_2 asphyxiation. The GIT was dissected out and photographed (a), the caecum isolated and weighed. Caeca were dried for 7 days at 37°C and weighed again to calculate the total percentage water lost to evaporation (b). Photographs of intestines were scored by assessors to determine the presence or absence of diarrhoea (c). Panel b presents mean percentages (+ SEM) for all 5 mice per experimental group. Panel c presents mean % fluid in caecum (+ SEM) for all 5 mice per experimental group. SBC (Solution) versus CTB (Solution) versus CT+CTB (Solution) versus CTB (SmPill) versus CT + CTB (SmPill), ns not significant.

Sample ID	*V. cholerae* common antigen	Inaba LPS
SmPills with *V. cholerae* (2.5x10⁸ cells)		
SmPills with *V. cholerae* + α-Galcer (2.5x10⁸ cells)		
Control sample with *V. cholerae* (1x10⁹ cells)		

Figure 4.5.7 – Accurate and intact loading of whole cell killed bacterial antigens into SmPills is possible. SmPills containing 2.5×10^8 JS1569 with or without α-Galcer were dissolved in PBS. Dot blots against rabbit anti-*V.cholerae* common antigen and Inaba-LPS were performed on the SmPill supernatants. Control sample corresponding to $\sim 1 \times 10^9$ Inaba *V.cholerae* from the same batch as those used in the SmPills is used as a standard for bacterial counts and intact LPS. Anti-*V.cholerae* common antigen dot blots were used to estimate bacterial numbers and Inaba-LPS dot blots were used to determine antigen stability. (Experiment performed by Stefan Karlsson, University of Gothenburg, Sweden.)



Figures 4.5.8 – SmPills enhance the ability of whole cell killed cholera and α-Galcer to enhance faecal IgA. C57BL/6 mice were vaccinated orally as per figure 4.5.1 with SBC as a control or JS1569 (2.5×10^8 bacteria per mouse) alone or with α-GalCer (10μg) in SBC solution or in SmPills. Faecal pellets were collected on days 13, 27 and 41. Inaba LPS - specific IgA antibody titres were determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. *V.cholerae* - α-Galcer (Solution) versus *V.cholerae* + α-Galcer (Solution) versus *V.cholerae* - α-Galcer (SmPill) versus *V.cholerae* + α-Galcer (SmPill), * $p<0.05$, ns, not significant. Panel d presents mean titres (+ SEM) for 5 mice per experimental group at each time point. The graph superimposed in d presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. *V.cholerae* - α-Galcer (Solution) versus *V.cholerae* + α-Galcer (Solution) versus *V.cholerae* - α-Galcer (SmPill) versus *V.cholerae* + α-Galcer (SmPill), * $p<0.05$, ns, not significant.

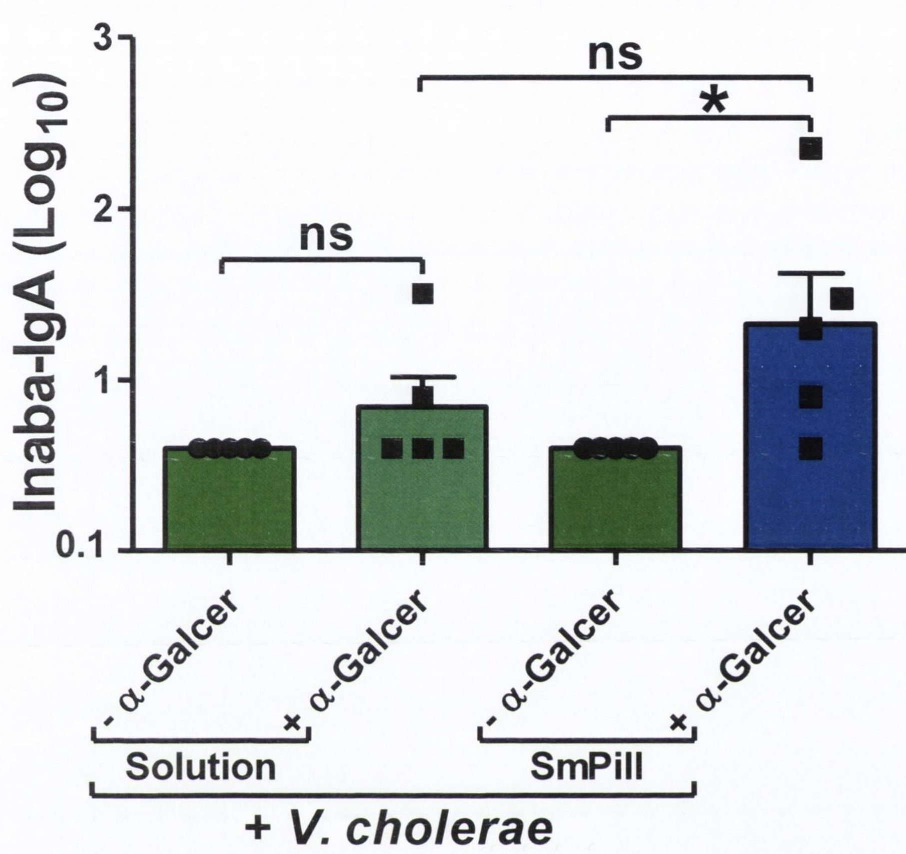


Figure 4.5.9 – Local mucosal immune responses are enhanced when whole cell killed cholera are delivered in SmPills together with α-Galcer. C57BL/6 mice were vaccinated orally as per figure 4.5.1 with SBC as a control or JS1569 (2.5×10^8 bacteria per mouse) alone or with α-GalCer (10μg) in SBC solution or in SmPills. On day 42, mice were sacrificed by cervical dislocation and perfused with heparin PBS. 3cm of the small intestines were dissected out and treated with saponin to extract the inter-tissue antibodies. Inaba LPS-specific IgA antibody titres in the supernatants were determined by end-point ELISA. Panel presents mean titres (+ SEM) for 5 mice per experimental group. *V.cholerae* - α-Galcer (Solution) versus *V.cholerae* + α-Galcer (Solution) versus *V.cholerae* - α-Galcer (SmPill) versus *V.cholerae* + α-Galcer (SmPill), * $p<0.05$, ns, not significant.

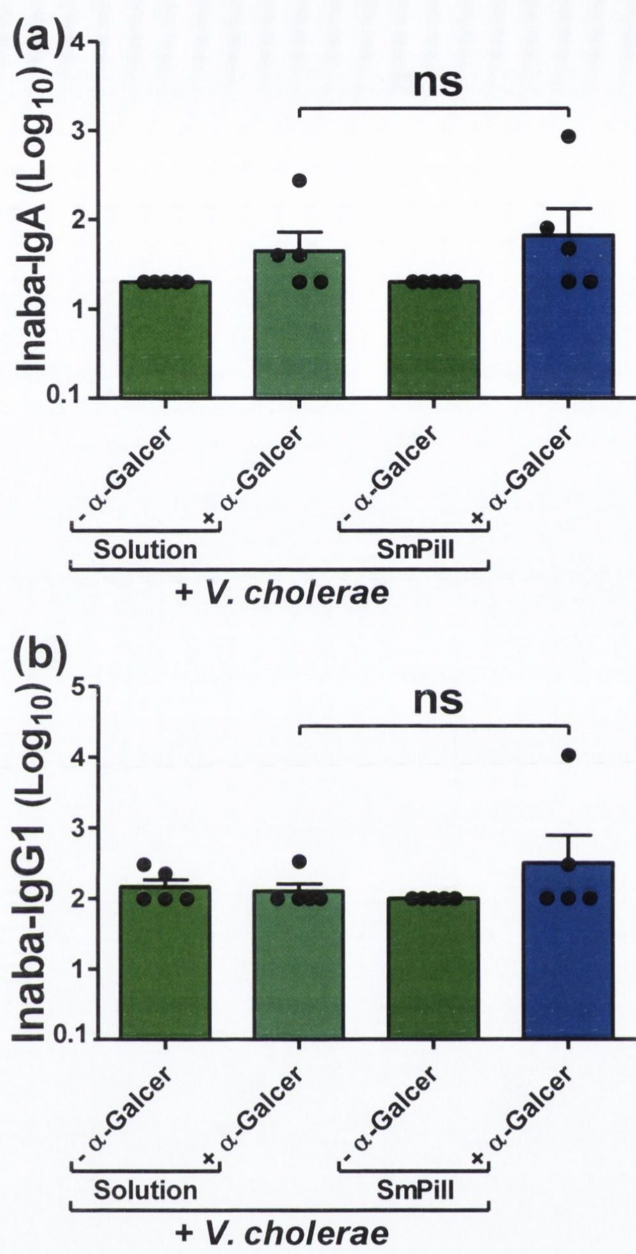


Figure 4.5.10 – No enhancement of serum antibody responses following oral vaccination was detected in mice vaccinated with SmPills containing whole cell killed and α-Galcer. C57BL/6 mice were vaccinated orally as per figure 4.5.1 with SBC as a control or JS1569 (2.5×10^8 bacteria per mouse) alone or with α-GalCer (10µg) in SBC solution or in SmPills. Serum was recovered on day 41 and Inaba LPS–specific IgA (a) and IgG1 (b) antibody titres determined by end-point ELISA. Panels a and b present mean titres (+ SEM) for 5 mice per experimental group. *V.cholerae* - α-Galcer (Solution) versus *V.cholerae* + α-Galcer (Solution) versus *V.cholerae* - α-Galcer (SmPill) versus *V.cholerae* + α-Galcer (SmPill), ns, not significant.

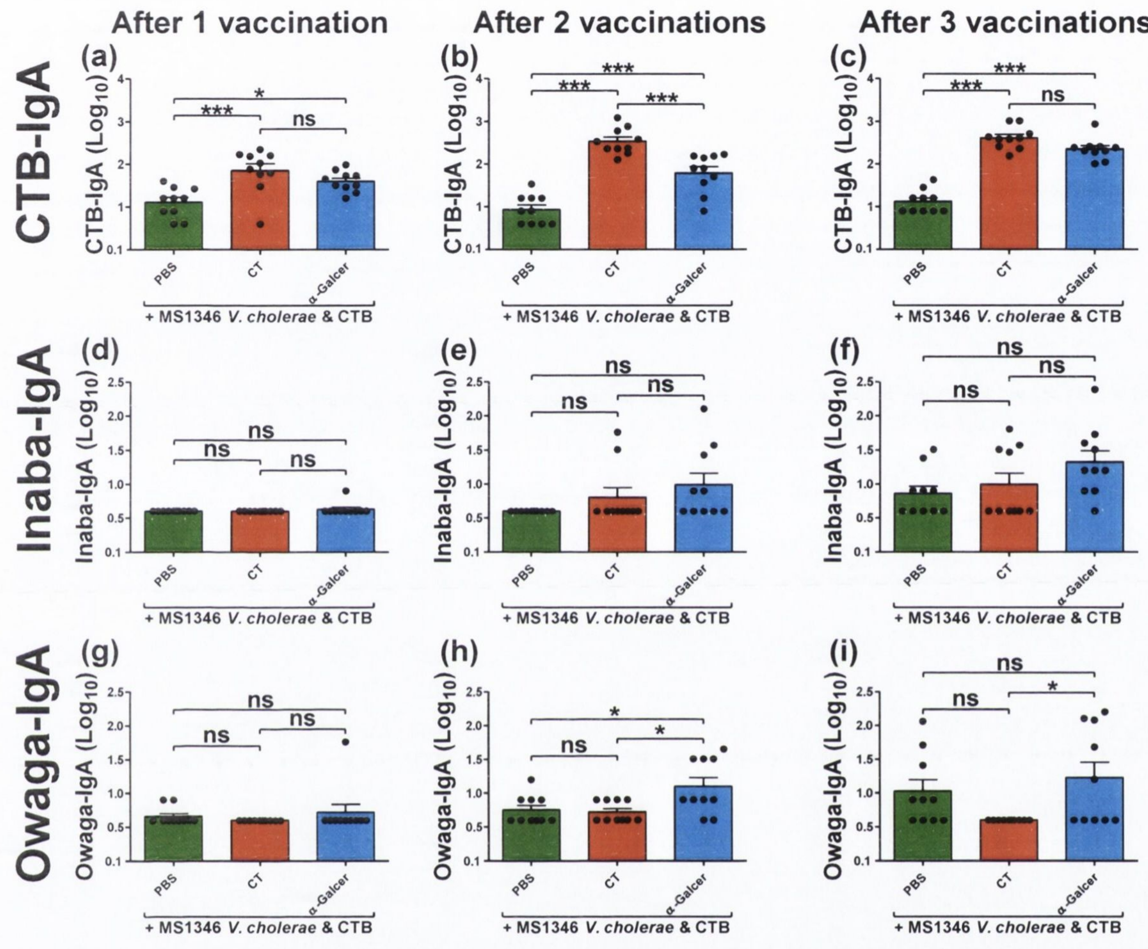


Figure 4.5.11 – Orally delivered α-Galcer and CT display differential adjuvant properties which are dependent on the physical properties of the specific antigen. C57BL/6 mice were immunised orally as per figure 4.5.1. PBS was used as a control while treatment groups included mice vaccinated with CTB (27.5μg per mouse) and MS1346 *V.cholerae* ($3x10^8$ Bacteria per mouse) either with or without CT (10μg) or α-Galcer (10μg) as an adjuvant. Faecal pellets were collected on days 13, 27 and 41. CTB-specific (a, b, & c), Inaba LPS–specific (d, e & f) and Ogawa LPS-specific (g, h & i) IgA antibody titres were determined by end-point ELISA. Panels a-i present mean titres (+ SEM) for all 10 mice per experimental group. PBS + CTB + *V.cholerae* versus CT + CTB + *V.cholerae* versus α-Galcer + CTB + *V.cholerae*. * p<0.05, ** p<0.01, *** p<0.001, ns, not significant.

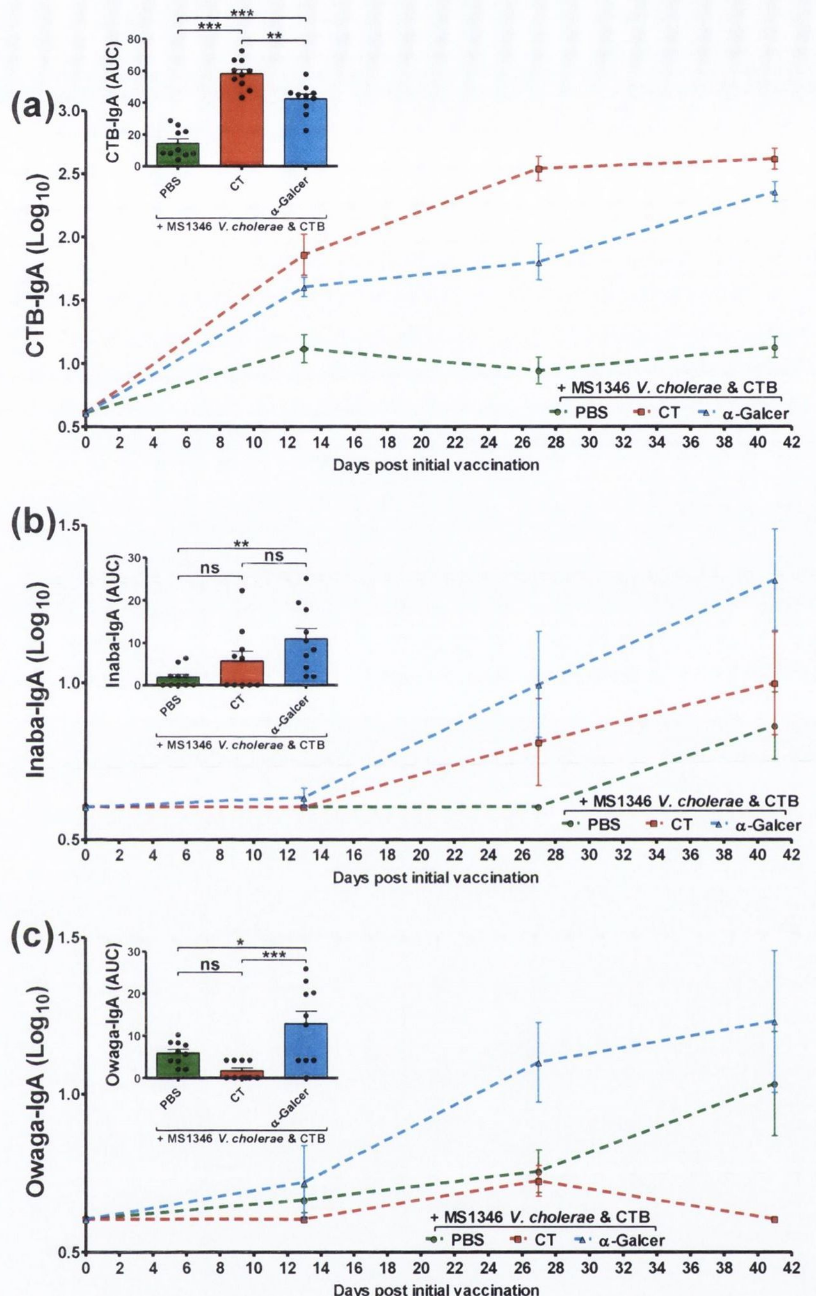


Figure 4.5.12 – α-Galcer and CT differentially enhance antigen-specific faecal IgA induction against different types of antigen. C57BL/6 mice were immunised orally as per figure 4.5.1. PBS was used as a control while treatment groups included mice vaccinated with CTB (27.5µg per mouse) and MS1346 *V.cholerae* (3x10^8 Bacteria per mouse) either with or without CT (10µg) or α-Galcer (10µg) as an adjuvant. Faecal pellets were collected on days 13, 27 and 41. CTB-specific (a), Inaba LPS–specific (b) and Ogawa LPS-specific (c) IgA antibody titres were determined by end-point ELISA. Panels a-c present mean titres across all three time-points (+ SEM) for 10 mice per experimental group at each time point. The graphs superimposed in a-c presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. PBS + CTB + *V.cholerae* versus CT + CTB + *V.cholerae* versus α-Galcer + CTB + *V.cholerae*. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns, not significant.

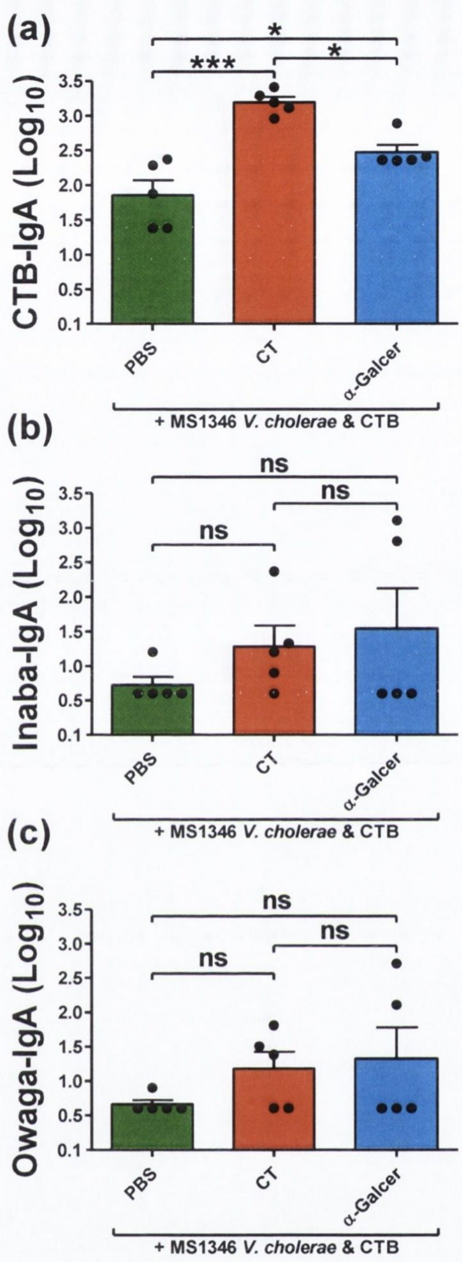


Figure 4.5.13 – CT is a more potent mucosal adjuvant for driving anti-CTB IgA than α-Galcer in the intestine. C57BL/6 mice were immunised orally as per figure 4.5.1. PBS was used as a control while treatment groups included mice vaccinated with CTB (27.5μg per mouse) and MS1346 *V.cholerae* ($3x10^8$ Bacteria per mouse) either with or without CT (10μg) or α-Galcer (10μg) as an adjuvant. On day 42, mice were sacrificed by cervical dislocation and perfused with heparin PBS. 3cm of the small intestines were dissected out and treated with saponin to extract the inter-tissue antibodies. CTB-specific (a), Inaba LPS–specific (b) and Ogawa LPS-specific (c) IgA antibody titres in the supernatants were determined by end-point ELISA. Panels a-c presents mean titres (+ SEM) for all 5 mice per experimental group. PBS + CTB + *V.cholerae* versus CT + CTB + *V.cholerae* versus α-Galcer + CTB + *V.cholerae*. * $p<0.05$, *** $p<0.001$, ns, not significant.

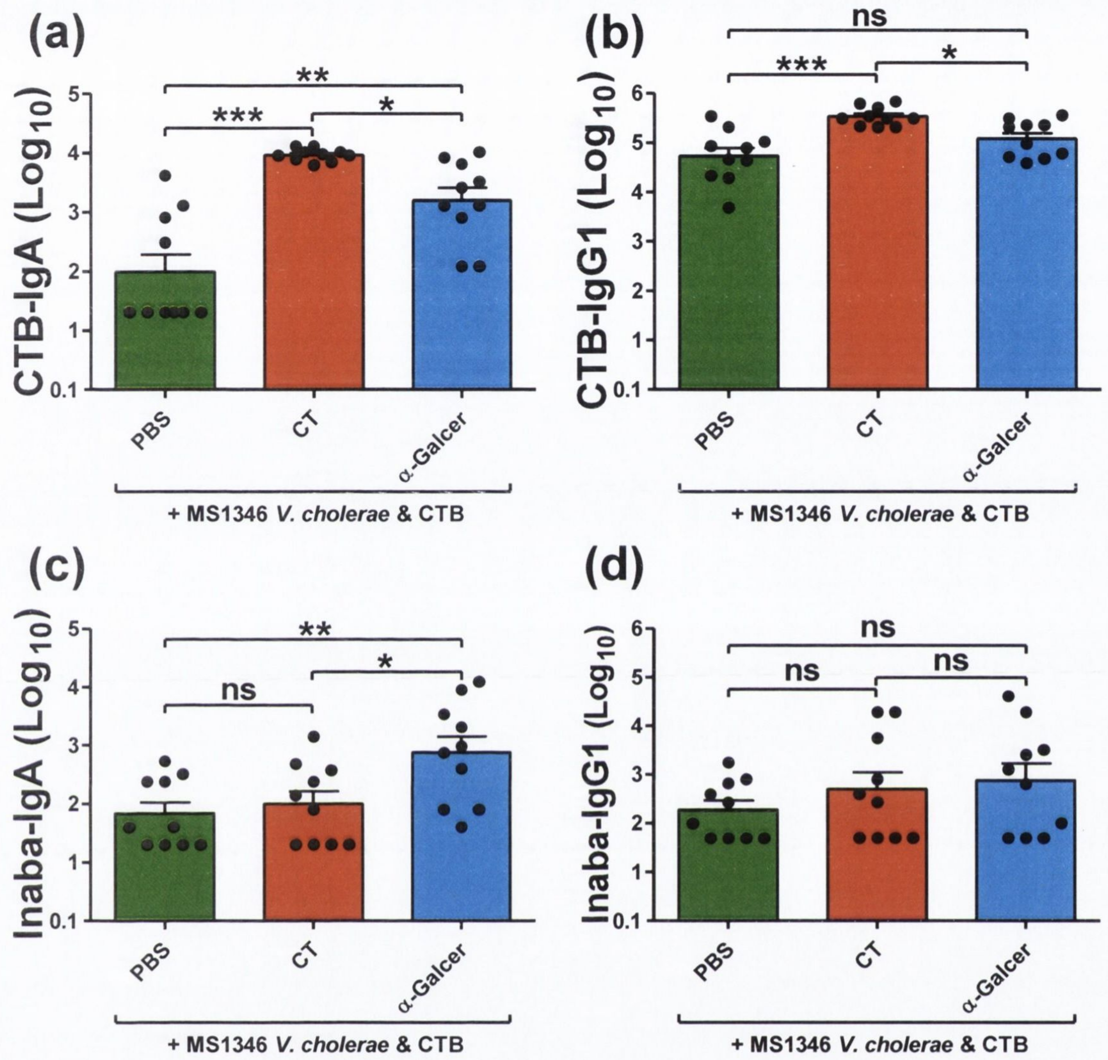


Figure 4.5.14 – α-Galcer promotes higher serum IgA titres against particulate antigens compared to CT after oral vaccination. C57BL/6 mice were immunised orally as per figure 4.5.1. PBS was used as a control while treatment groups included mice vaccinated with CTB (27.5µg per mouse) and MS1346 *V.cholerae* ($3x10^8$ Bacteria per mouse) either with or without CT (10µg) or α-Galcer (10µg) as an adjuvant. Serum on day 41 and CTB-specific and Inaba LPS–specific IgA (a&c) and IgG1 (b&d) antibody titres determined by end-point ELISA. Panels a-d present mean titres (+ SEM) for all 10 mice per experimental group. PBS + CTB + *V.cholerae* versus CT + CTB + *V.cholerae* versus α-Galcer + CTB + *V.cholerae*. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns, not significant.

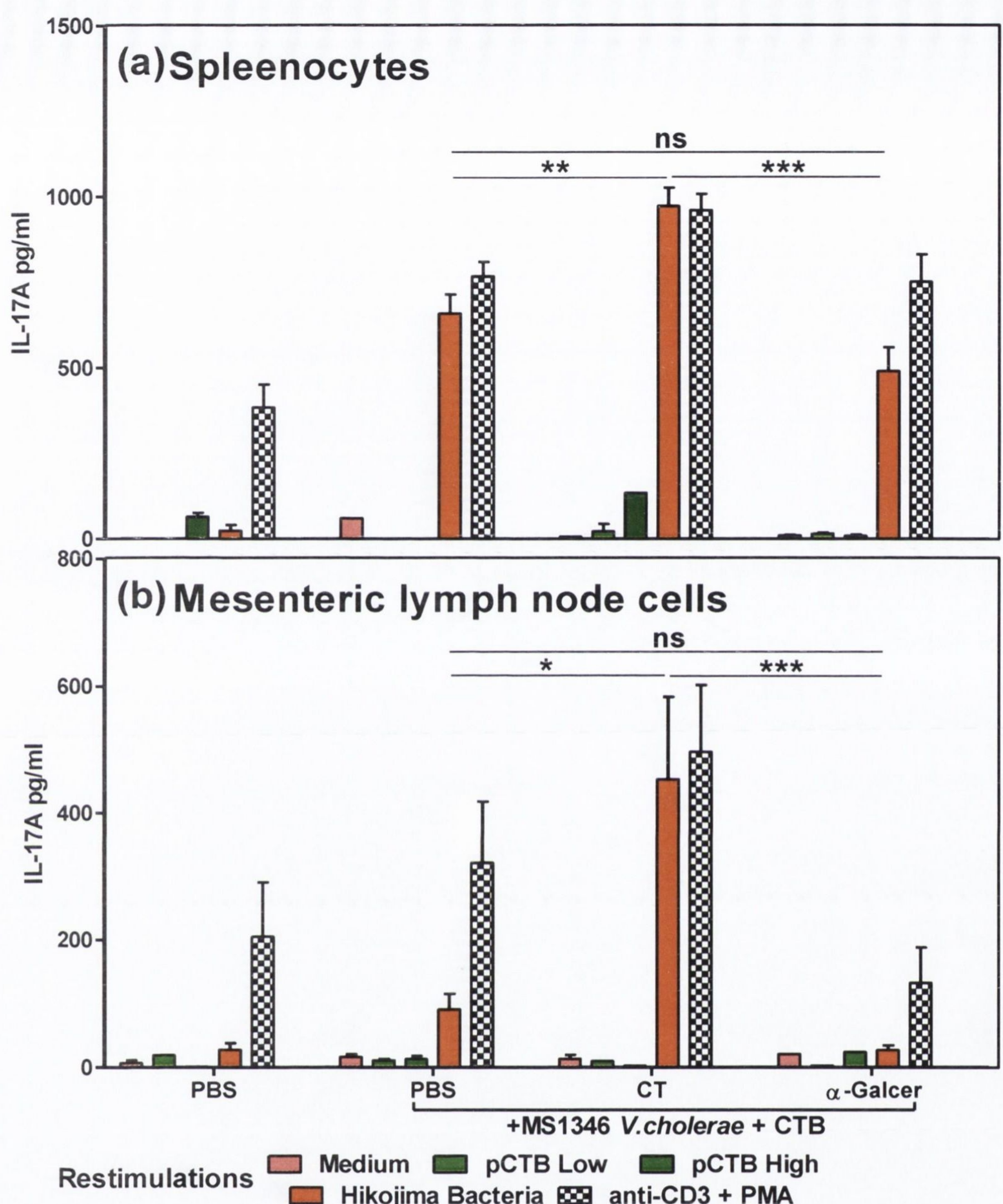


Figure 4.5.15 – CT promotes stronger antigen-specific Th17 responses than α-Galcer. C57BL/6 mice were immunised orally as per figure 4.5.1. PBS was used as a control while treatment groups included mice vaccinated with CTB (27.5μg per mouse) and MS1346 *V.cholerae* ($3x10^8$ Bacteria per mouse) either with or without CT (10μg) or α-Galcer (10μg) as an adjuvant. The mice were sacrificed on day 42. Spleens (a) and MLNs (b) were recovered and restimulated *ex vivo* with T cell medium, pCTB Low (10μg/ml), pCTB High (50μg/ml), Hikojima Bacteria (FK MS1346 *V.cholerae* (1 bacteria/cell)) or anti-CD3 (0.5μg/ml) in combination with PMA (25ng/ml). After 72 hours, supernatants were collected and analysed for the cytokine IL-17 by ELISA. Panels a & b present mean cytokine concentrations (+ SEM) for 5 mice per experimental group tested individually in triplicate. PBS + CTB + *V.cholerae* versus CT + CTB + *V.cholerae* versus α-Galcer + CTB + *V.cholerae*. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns, not significant.

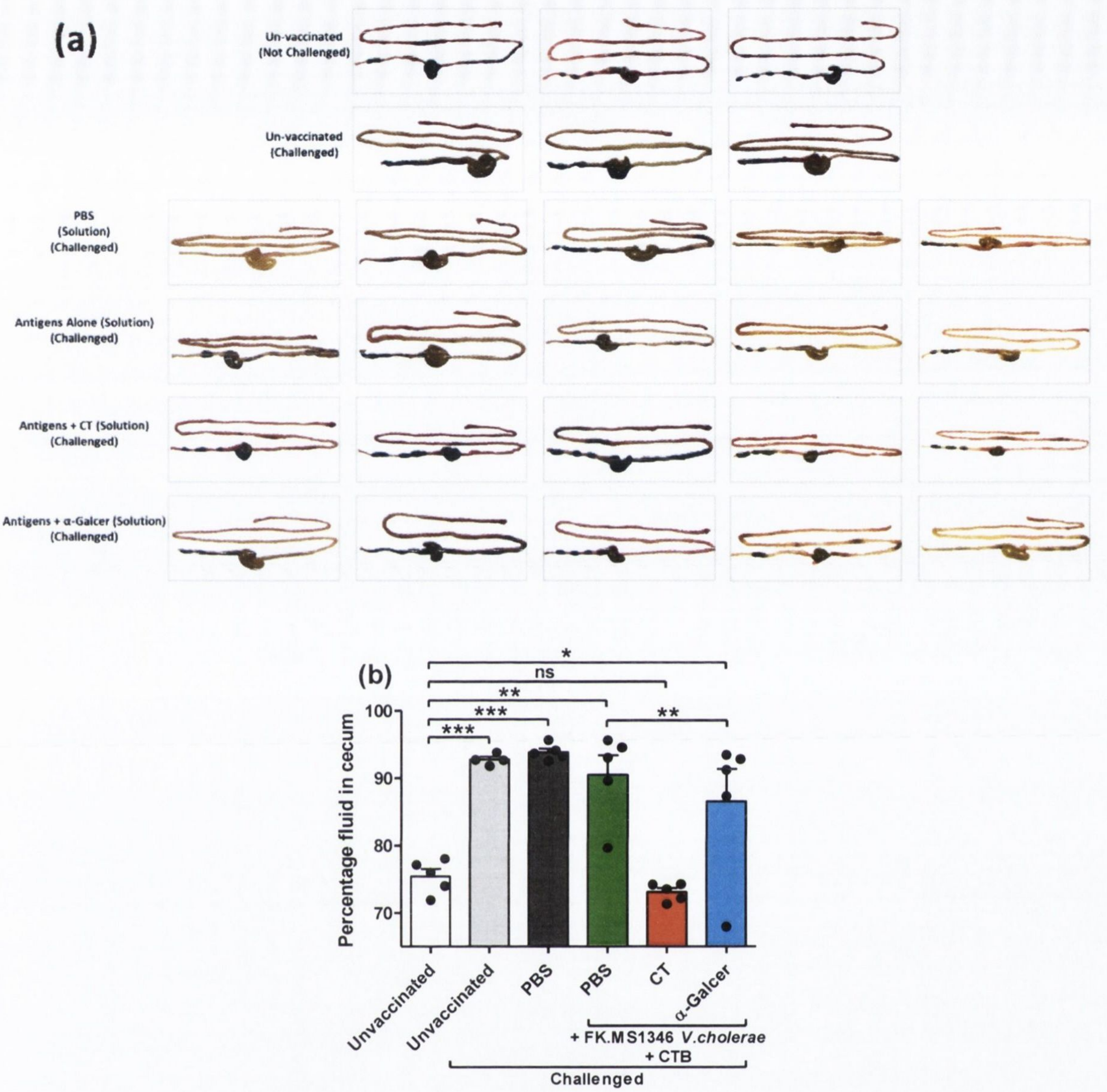


Figure 4.5.16 – CT promotes a stronger protective immune response against oral cholera toxin challenge than α-Galcer. C57BL/6 mice were immunised orally as per figure 4.5.1. SBC was used as a control while treatment groups included mice vaccinated with CTB (27.5μg per mouse) and MS1346 *V.cholerae* (3x10^{8} Bacteria per mouse) either with or without CT (10μg) or α-Galcer (10μg) as an adjuvant. On day 42, mice were orally challenged with CT (20μg per mouse). After 6 hours mice were sacrificed by cervical dislocation. The GIT was dissected out and photographed (a), the caecum isolated and weighed. Caeca were dried for 7 days at 37°C and weighed again to calculate the total percentage water lost to evaporation (b). Panel b presents mean percentages (+ SEM) for all 5 mice per experimental group. Unvaccinated (Unchallenged) versus Unvaccinated (Challenged) versus PBS (Challenged) versus PBS + CTB + *V.cholerae* (Challenged) versus CT + CTB + *V.cholerae* (Challenged) versus α-Galcer + CTB + *V.cholerae* (Challenged). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns, not significant.

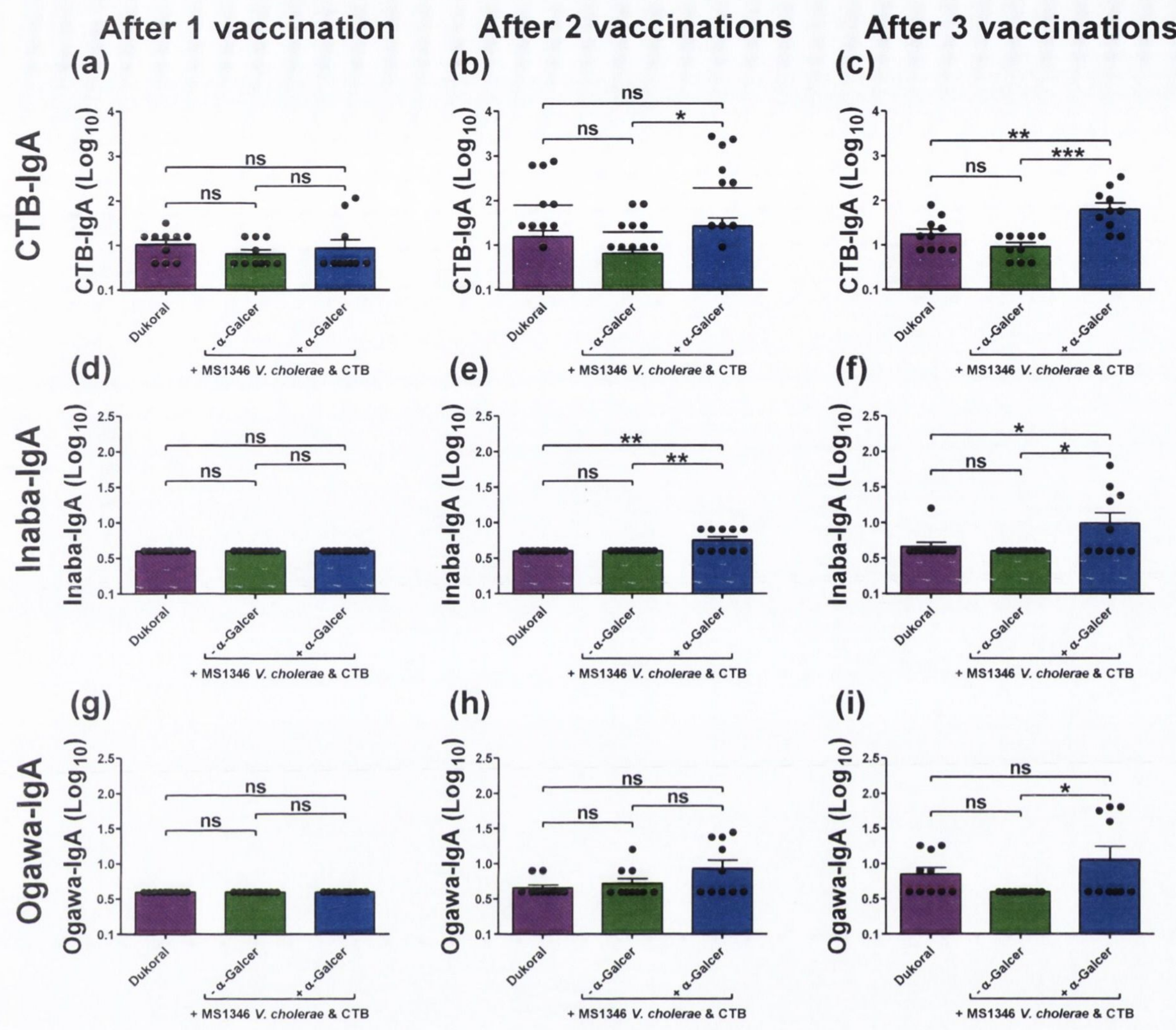


Figure 4.5.17 – SmPills containing whole cell killed cholera together with CTB and α-Galcer elicit stronger faecal antigen-specific IgA titres than Dukoral®. C57BL/6 mice were immunised orally as per figure 4.5.1. PBS was used as a control while treatment groups included mice vaccinated with Dukoral® (27.5μg CTB and $3x10^8$ Bacteria per mouse) in solution or with SmPills containing CTB (27.5μg per mouse) and MS1346 *V.cholerae* ($3x10^8$ Bacteria per mouse) with or without α-Galcer (10μg) as an adjuvant. Faecal pellets were collected on days 13, 27 and 41. CTB-specific (a, b, & c), Inaba LPS–specific (d, e & f) and Ogawa LPS-specific (g, h & i) IgA antibody titres were determined by end-point ELISA. Panels a-i present mean titres (+ SEM) for 10 mice per experimental group. Dukoral® (Solution) versus CTB + *V.cholerae* - α-Galcer (SmPill) versus α-Galcer + CTB + *V.cholerae* (SmPill), * p<0.05, ** p<0.01, *** p<0.001, ns, not significant.

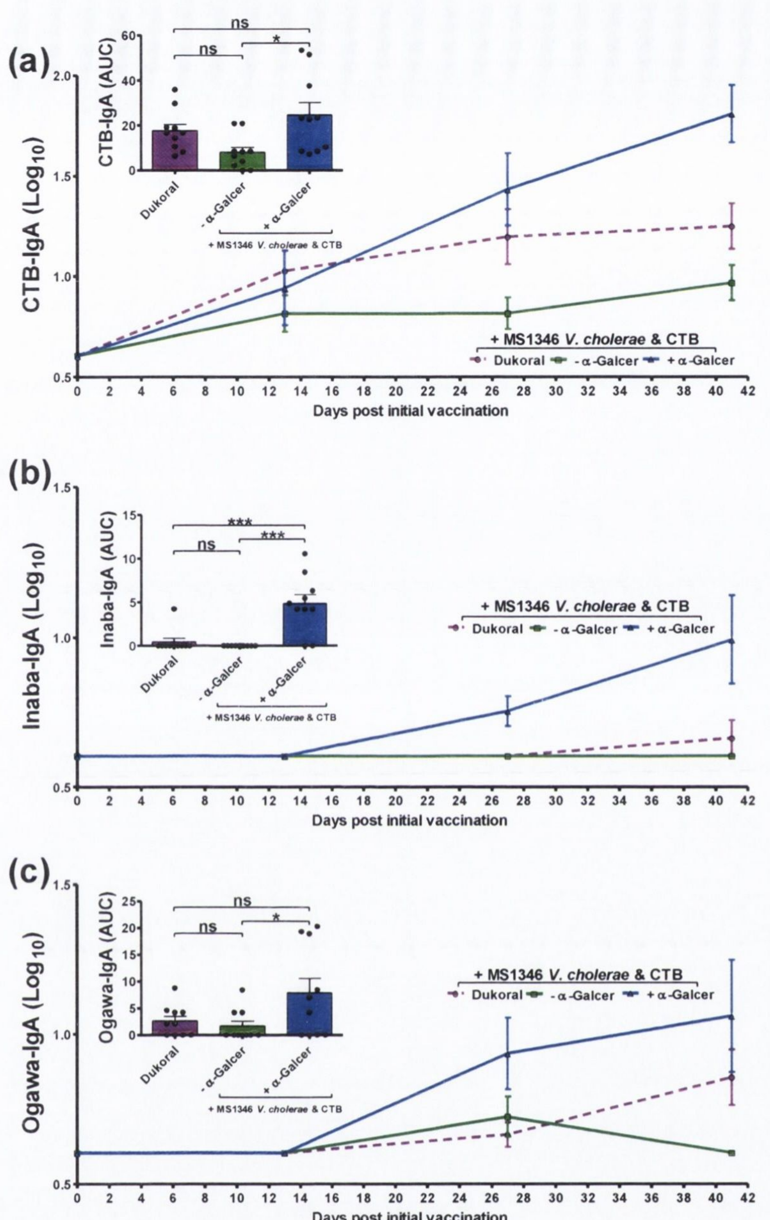


Figure 4.5.18 – Stronger faecal IgA induction is observed after oral vaccination SmPills containing whole cell killed cholera together with CTB and α-Galcer compared Dukoral®. C57BL/6 mice were immunised orally as per figure 4.5.1. PBS was used as a control while treatment groups included mice vaccinated with Dukoral® (27.5μg CTB and 3×10^8 Bacteria per mouse) in solution or with SmPills containing CTB (27.5μg per mouse) and MS1346 *V.cholerae* (3×10^8 Bacteria per mouse) with or without α-Galcer (10μg) as an adjuvant. Faecal pellets were collected on days 13, 27 and 41. CTB-specific (a, b, & c), Inaba LPS–specific (d, e & f) and Ogawa LPS-specific (g, h & i) IgA antibody titres were determined by end-point ELISA. Panels a-c present mean titres across all three time-points (+ SEM) for 10 mice per experimental group at each time point. The graphs superimposed in a-c presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. Dukoral® (Solution) versus CTB + *V.cholerae* - α-Galcer (SmPill) versus α-Galcer + CTB + *V.cholerae* (SmPill), * $p<0.05$, *** $p<0.001$, ns, not significant.

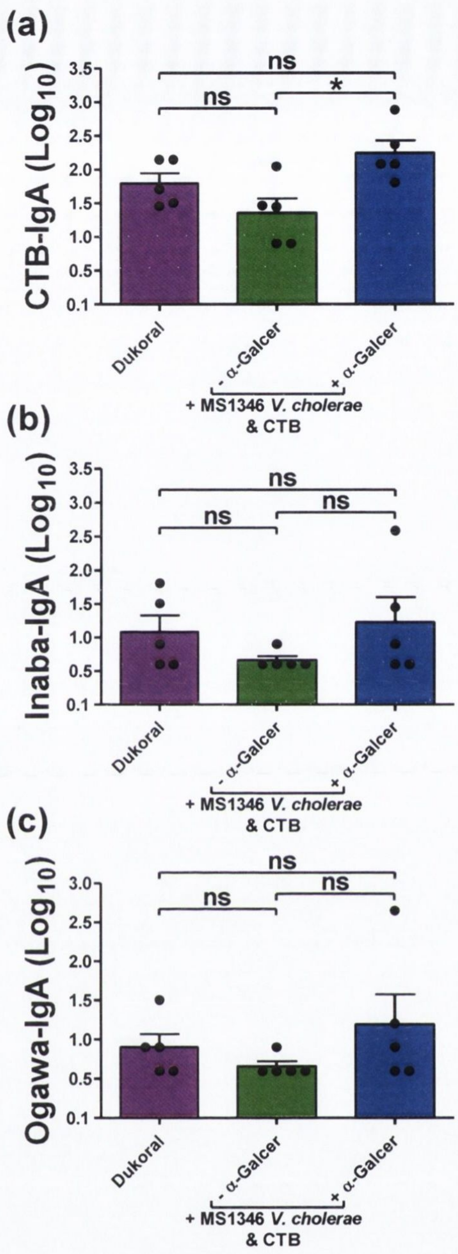


Figure 4.5.19 – Intestinal tissue antigen-specific IgA titres comparable after oral vaccination with either Dukoral® or whole cell killed cholera, CTB and α-Galcer delivered in SmPills. C57BL/6 mice were immunised orally as per figure 4.5.1. PBS was used as a control while treatment groups included mice vaccinated with Dukoral® (27.5μg CTB and $3x10^8$ Bacteria per mouse) in solution or with SmPills containing CTB (27.5μg per mouse) and MS1346 *V.cholerae* ($3x10^8$ Bacteria per mouse) with or without α-Galcer (10μg) as an adjuvant. On day 42, mice were sacrificed by cervical dislocation and perfused with heparin PBS. 3cm of the small intestines were dissected out and treated with saponin to extract the inter-tissue antibodies. CTB-specific (a), Inaba LPS–specific (b) and Ogawa LPS-specific (c) IgA antibody titres in the supernatants were determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for 5 mice per experimental group. Dukoral® (Solution) versus CTB + *V.cholerae* - α-Galcer (SmPill) versus α-Galcer + CTB + *V.cholerae* (SmPill), * $p<0.05$, *** $p<0.001$, ns, not significant.

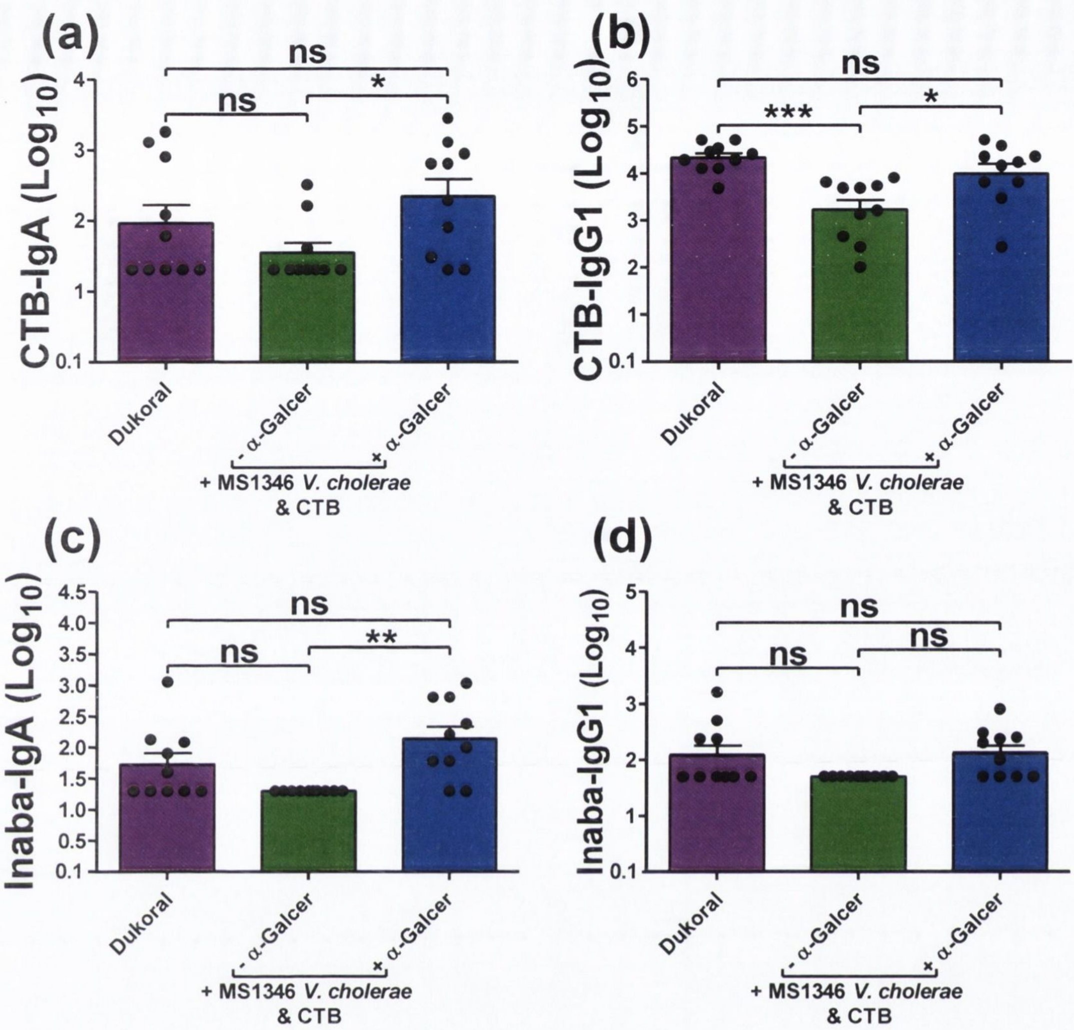


Figure 4.5.20 – Serum antigen-specific IgA titres are not significantly different after oral vaccination with either SmPills loaded with whole cell killed cholera, CTB and α-Galcer or Dukoral®. C57BL/6 mice were immunised orally as per figure 4.5.1. PBS was used as a control while treatment groups included mice vaccinated with Dukoral® (27.5μg CTB and $3x10^8$ Bacteria per mouse) in solution or with SmPills containing CTB (27.5μg per mouse) and MS1346 *V.cholerae* ($3x10^8$ Bacteria per mouse) with or without α-Galcer (10μg) as an adjuvant. Serum was recovered on day 41. CTB-specific and Inaba LPS–specific IgA (a & c) and IgG1 (b & d) antibody titres in the serum were determined by end-point ELISA. Panels a-d present mean titres (+ SEM) for 10 mice per experimental group. Dukoral® (Solution) versus CTB + *V.cholerae* (SmPill) versus α-Galcer + CTB + *V.cholerae* (SmPill), * p<0.05, ** p<0.01, *** p<0.001, ns, not significant.

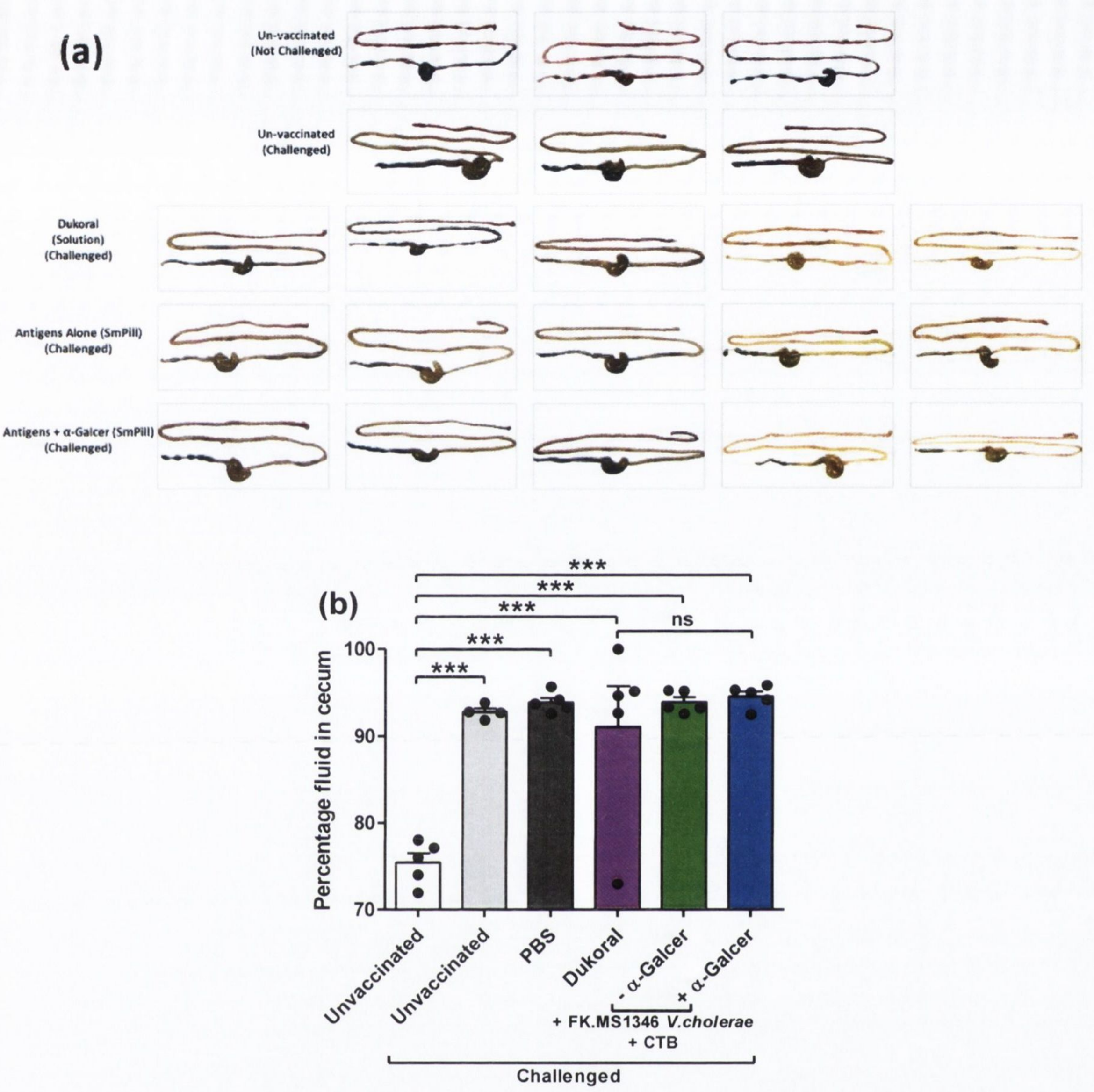


Figure 4.5.21 – No significant protection against oral cholera toxin challenge was detected after vaccination with either Dukoral® or whole cell killed cholera together with CTB and α-Galcer loaded into SmPills. C57BL/6 mice were immunised orally as per figure 4.5.1. PBS was used as a control while treatment groups included mice vaccinated with Dukoral® (27.5μg CTB and $3x10^8$ Bacteria per mouse) in solution or with SmPills containing CTB (27.5μg per mouse) and MS1346 *V.cholerae* ($3x10^8$ Bacteria per mouse) with or without α-Galcer (10μg) as an adjuvant. On day 42, mice were orally challenged with CT (20μg per mouse). After 6 hours mice were sacrificed by cervical dislocation, the GIT was dissected out, the intestine was photographed and caecum isolated and weighed. Petri dishes containing the caecum were placed in an oven for 7 days at 37°C and weighed again to calculate the total percentage water lost to evaporation (b). Panel b presents mean percentages (+ SEM) for all 5 mice per experimental group. Unvaccinated (Unchallenged) versus Unvaccinated (Challenged) versus PBS (Solution) versus Dukoral® (Solution) versus CTB + *V.cholerae* - α-Galcer (SmPill) versus α-Galcer + CTB + *V.cholerae* (SmPill), *** $p<0.001$, ns, not significant.

Chapter Five

Determining the contribution of IL-17 to the protection against oral cholera toxin challenge mediated by oral subunit vaccines

5.1 – Introduction

While the benefits of oral vaccination are many, current protocols for evaluating the efficacy of candidate oral vaccines require multiple rounds of vaccination which is both costly and time consuming, often lasting over 2 months to conduct a single study (Figure 5.5.1). This places significant obstacles in the path to developing a rapid and cost effective method for screening prototype oral vaccine formulations. These challenges further compound one of the greatest challenges facing oral vaccination which is the relatively low immunogenicity of orally delivered subunit antigens and the lack of effective and safe mucosal adjuvants [12]. Although limited, *in vitro* DC assays can be used to rapidly screen adjuvants and vaccine formulations for their ability to induce DC maturation and cytokine production, which can provide some indication of their immunomodulatory potential, however follow up *in vivo* studies are required to assess the true immune-potentiating capacity of an adjuvant [351]. Isolating and culturing intestinal immune cells is arduous, expensive and time consuming and often results in low yields of viable purified cells [352] [353]. Identification of a mucosal tissue biomarker indicative of mucosal adjuvant efficacy would be very valuable. Herein, the potential of IL-17 as a biomarker for mucosal adjuvanticity was investigated.

The labile toxins CT and LT are currently the most potent mucosal adjuvants available [146]. While the toxicity of the native molecules restricts their application in humans their use as model mucosal adjuvants and tools to dissect mucosal immune responses in basic research is invaluable. CT has also been shown to be capable of inducing memory Th17 responses following oral vaccination [354, 355] and Th17 cells have been implicated in vaccine-mediated protection against mucosal pathogens while the important role of the IL-17 family of cytokines in mucosal homeostatis and protective mucosal immunity has been documented [66, 79]. Furthermore, the mucosal adjuvanticity of CT was shown to be highly dependent on

IL-17A [355]. Adjuvant screening studies performed by McNeela *et al* revealed that CT but not soluble TLR agonists had the capacity to elicit strong antigen-specific type-17 responses after oral vaccination (Fig 5.1.1). Furthermore, CT and not α-Galcer elicited strong IL-17A production from splenocytes and mesenteric lymph node (MLN) cells upon re-stimulation with formalin killed *V.cholerae* following oral vaccination against these (Figure 4.5.15)

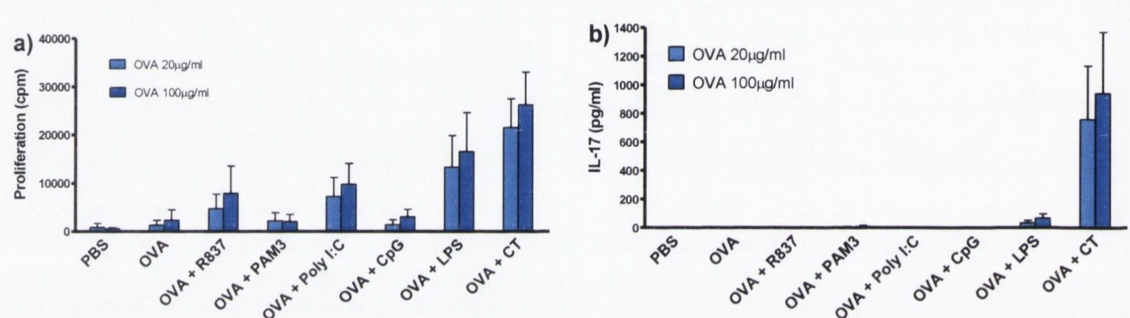


Figure 5.1.1 – Cholera toxin is a potent oral adjuvant for eliciting antigen specific Th17 responses following oral vaccination. BALB/c mice were immunized orally on 3 consecutive days with ovalbumin (100 μg/mouse) either alone or with R837 (50μg), Pam3Csk (50μg), PolyI:C (50μg), CpG (50μg), LPS (50μg) or CT (10μg) and were boosted on week 4 with an identical series of immunizations. One week following the final immunization, splenocytes were recovered and stimulated *in vitro* with a range of concentrations of antigen indicated in each (Figure. (a) Proliferative responses were assessed by [^{3}H]-thymidine incorporation after 4 days incubation. (b) IL-17 concentrations in the supernatants were determined by ELISA after 3 days. (Experiment performed by Edel McNeela, unpublished data)

Oral cholera toxin challenge (OCTC) (introduced in section 4.3.1 and described in section 2.2.5) is an accepted model for evaluating the protective efficacy of oral cholera vaccines (OCVs) with a toxin component [339]. It is also a useful model to dissect the involvement of specific pathways and cytokines that drive anti-toxin immunity. Research into CTB-expressing rice (muco-rice) by Tokuhara *et al* determined that protection against OCTC was dependent on SIgA [340]. Mice lacking the polymeric Ig receptor (pIgR) were not protected by the vaccine, while wild type mice were [340]. As discussed in section 1.7.1, pIgR is a

receptor required for the transport of IgA across intestinal epithelial cells (IECs) into the lumen where it is secreted as SIgA [83]. This study highlighted the important role of pIgR in vaccine mediated protection against oral CT and LT challenge [340].

Recently, a paper was published by Cao *et al*, which showed that the concentrations of SIgA in the lumen of IL-17R$^{-/-}$ mice were significantly lower than in wild type animals [356]. Furthermore this defect in SIgA secretion was linked to reduced basal pIgR expression in these mice [356]. This study showed that Th17 responses in the gut can enhance SIgA secretion by augmenting both IgA production by B cells and directly increasing SIgA secretion by epithelial cells by up-regulating pIgR expression on IECs (Figure 5.1.2). This study however, only examined the significance of Th17 responses on SIgA secretion in the context of intestinal homeostatis [356].

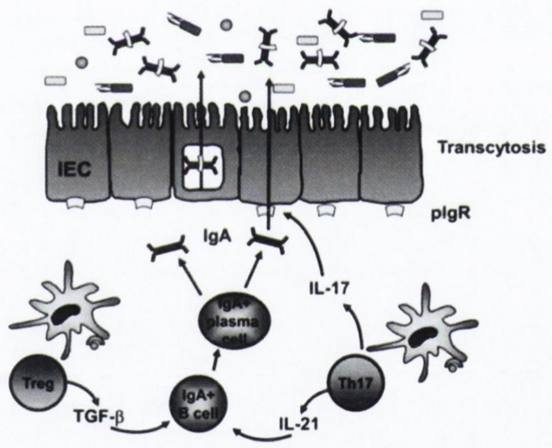


Figure 5.1.2 – IL-17 and IL-21 secreted by Th17 cells signals through epithelial cells to up-regulate pIgR and through B cells to enhance differentiation to plasma cells which secrete high levels of IgA. Coordinated secretion of IL-21 and TGF-β by gut Th17 and Treg cells respectively drives the differentiation of IgA^{+} B cells into IgA secreting plasma cells. Simultaneously IL-17 produced by Th17 cells acts to up regulate the expression of pIgR on gut epithelial cells, to which dimeric IgA binds. This bound IgA is transcytosed into the lumen of the intestine where it is released with the secretory component of pIgR attached. The combined effect of increased B cell differentiation into plasma cells and up regulated pIgR expression on epithelial cells enhances the rate of SIgA secretion into the lumen of the gut. (Taken from Cao, A.T. 2012)

Concentrations of IL-17 were significantly elevated in extracts from the small intestine following CT delivery, suggesting that rapid production (within 6 hours of administration) of IL-17 in the gut could be a viable biomarker for the efficacy of a candidate oral adjuvant (Figure 5.1.3). The observations made in the afore-mentioned studies served as the foundation for the work conducted in this chapter.

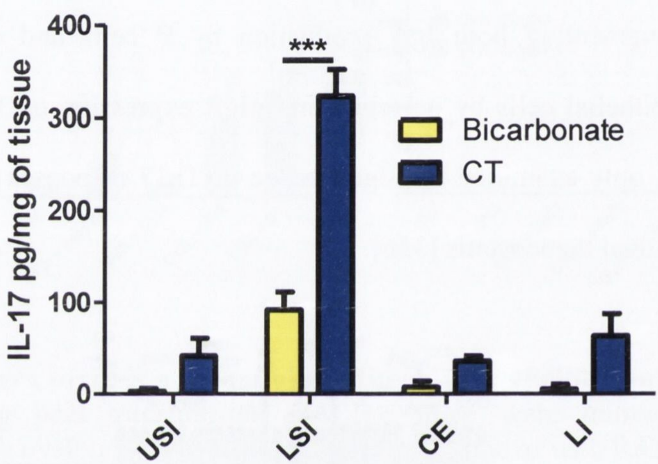


Figure 5.1.3 – Oral administration of CT induces local IL-17 production. BALB/c mice were administered either SBC or 10μg CT or by oral gavage. 6 hours post later, mice were sacrificed; the intestines were removed, washed and separated into Upper Small Intestine (USI), Lower Small Intestine (LSI), Caecum (CE) and Large Intestine (LI). Tissue samples were placed into a protease inhibitor cocktail and homogenized. IL-17 production in intestinal regions was measured 6 hours post-administration by ELISA. Cytokine concentrations are expressed as pg per mg of intestinal tissue. (McEntee, Davitt and McNeela; *unpublished*)

5.2 – Hypothesis, Aims and Objectives

- Hypothesis –

The rapid induction of intestinal IL-17 by oral vaccines is required for IgA secretion suggests an important role for this cytokine in mucosal immune responses and may provide a valuable biomarker for assessing mucosal adjuvants.

- Aims and Objectives –

1. To determine the effect of IL-17R deficiency on intestinal production and secretion of antigen-specific SIgA in response to an effective oral vaccine (CT and CTB).

2. To examine if IL-17R is required for vaccination with CT and CTB to provide protection against oral toxin challenge.

5.3 – Results

5.3.1 – IL-17R is required for secretion of antigen-specific IgA following oral vaccination.

IL-17 has been implicated in the regulation of intestinal homeostatis and immunity. One of the main components of mucosal immune function is SIgA. The central role of IL-17 in the establishment of homeostatic SIgA responses in mice has been established [356]. SIgA is primarily transported across intestinal epithelial cells by pIgR [83].

In order to determine the effect of IL-17R deficiency on the ability of oral vaccination to elicit SIgA responses, mice were orally immunised with SBC alone or CTB (20μg). Additionally, wild type (WT) C57BL/6 and IL-17R$^{-/-}$ mice were orally immunised with CTB (20μg) and CT (10μg) as an adjuvant as per figure 5.5.1. Faecal pellets were collected on days 13, 27, 41 and CTB-specific IgA titres determined by end-point ELISA (Figure 5.5.2).

Mice receiving CT and CTB displayed significantly elevated CTB-specific faecal IgA titres compared to mice receiving CTB alone (Figure 5.5.2). Significantly lower antigen-specific IgA titres were detected in faecal pellet supernatants after 1 (Figure 5.5.2 a), 2 (Figure 5.5.2 b) and 3 (Figure 5.5.2 c) rounds of oral vaccinations in IL-17R deficient mice compared to WT mice receiving CT and CTB. Furthermore, wildtype mice displayed a significant increase in the induction of antigen-specific IgA titres in faecal pellets compared to mice receiving CTB only (Figure 5.2.2 d). This effect was not observed when in IL-17R deficient mice where no significant increase in CTB-IgA induction was observed compared to mice vaccinated with CTB alone (Figure 5.5.2 d). Overall, IL-17R deficient mice showed a significantly lower induction of IgA during the course of the experiment compared to WT mice (Figure 5.2.2 d).

5.3.2 – IL-17R deficiency results in impaired local adaptive IgA responses in the tissue of the GIT.

IL-17 has been shown to directly influence SIgA secretion by up regulating pIgR but the cytokine was also shown to play an important role in promoting the development of IgA producing plasma cells [356].

To examine the role of IL-17R in the establishment of local vaccine induced intestinal IgA responses, mice were orally immunised as in 5.3.1. On day 42 mice were orally challenged with CT (20μg) and 6 hours later euthanized by CO_2 asphyxiation. The intestines were dissected out and divided into 14 sections (Figure 5.5.3 b). These sections were cleaned, placed into ice cold inhibition buffer and kept on ice. Saponin was added and left overnight to disrupt cell membranes and release antibody molecules into the supernatants. CTB-specific IgA titres in the supernatants were determined by end-point ELISA (Figure 5.5.3 a).

Oral vaccination with CT and CTB elicited high titres of antigen-specific IgA along the entire small and large intestine after OCTC (Figure 5.5.3 a). In contrast IL-17R deficient mice exhibited statistically significantly lower titres than WT mice when orally vaccinated with CT and CTB (Figure 5.5.3 a). While WT mice exhibited only small variations in titres along the length of the GIT, IL-17R deficient mice displayed a noticeable trend of reducing titres distending down the GIT towards the rectum, demonstrated by the reduction of significance in CTB-specific IgA titres between IL-17R deficient mice vaccinated with CT and CTB and WT mice vaccinated only with CTB (Figure 5.5.3 a). WT mice vaccinated with CT and CTB display a consistent trend of significantly higher CTB-specific IgA titres along the length of the GIT compared to mice vaccinated with CTB alone (Figure 5.3.3 a). Interestingly, a trend of increasing significance in CTB-specific IgA titres is observed in the lower portions of the GIT in WT mice versus IL-17R deficient mice vaccinated with CT and CTB (Figure 5.3.3 a).

5.3.3 – The defect in the induction of humoral immunity in the gut mucosa is restricted to local and not systemic immune responses following oral vaccination.

The role of IL-17 in the intestinal mucosa and its influence on vaccine-induced immunity has been characterised [66]. IL-17R$^{-/-}$ mice showed significantly lower faecal pellet IgA (Figure 5.5.2) and intestinal tissue IgA (Figure 5.5.3) after oral vaccination with CT and CTB compared to WT mice.

In order to determine if the defect in the induction of humoral immune responses following oral vaccination is restricted to the local mucosa or if there is also a defect in systemic responses blood was obtained from mice on day 41 after oral vaccination (see 5.3.1) and CTB-specific IgA (Figure 5.5.4 a), IgG1 (Figure 5.5.4 b), IgG2b (Figure 5.5.4 c) and IgG2c (Figure 5.5.4 d) antibody titres were determined in sera by end-point ELISA.

No significant differences in serum CTB specific IgA titres were detected between wild type and IL-17R deficient mice after oral vaccination with CT and CTB (Figure 5.5.4 a). Equally IgG1 titres were not significantly different between both WT and IL-17R$^{-/-}$ mice vaccinated with CT and CTB (Figure 5.5.4 b). There were also no significant differences detected in IgG2b and IgG2c serum CTB-specific titres between wild type and IL-17R deficient mice, with both groups exhibiting higher titres than mice vaccinated with CTB alone (Figure 5.5.4 c and d). WT and IL-17R$^{-/-}$ mice vaccinated with CT and CTB displayed significantly higher anti-CTB titres for IgA and all IgG subtypes when compared to mice vaccinated with CTB alone (Figure 5.5.4).

5.3.4 – An efficacious oral anti-toxic cholera vaccine loses its protective efficacy in the absence of the IL-17 receptor.

Mice lacking IL-17R were shown to have reduced IEC pIgR expression which translated to reduced total SIgA in the lumen of the intestine [356]. Mice deficient in pIgR, and thus SIgA in the lumen of the GIT were also susceptible to OCTC, despite being vaccinated with an efficacious vaccine [340]. When vaccinated with CT + CTB significantly higher CTB-specific IgA titres were detected in WT mice compared to IL-17R deficient mice (Figure 5.5.2 and 5.5.3).

In order to determine if a deficiency in IL-17R would compromise the induction of protective anti-toxin immunity in the GIT, mice were vaccinated (as per 5.3.1) and 42 days later orally challenged with CT (20μg) and 6 hours later euthanized by CO_2 asphyxiation. The intestines were dissected out, photographed (Figure 5.5.5 a) and caeca separated, weighed before being dried for 7 days and weighed again. The percentage of water lost to evaporation from the ceca was calculated as an additional measure of protection (Figure 5.5.5 b).

Mice receiving either SBC or CTB alone were not protected and succumbed to OCTC as seen by the presence of a large volume of infiltrating fluid in the intestinal tract (Figure 5.5.5 a). All WT mice vaccinated with CT and CTB were protected against CT challenge (Figure 5.5.5 c). This was determined by the presence of solid faecal matter in the caecum and small intestine, with no fluid accumulation (Figure 5.5.5 a). However, mice deficient in IL-17R that were vaccinated with CT and CTB were not protected when challenged. These mice displayed Diarrhoea symptoms identical to the unprotected mice in the SBC vaccinated group (Figure 5.5.5 a).

5.4 – Discussion

Efforts to develop new or improved oral vaccines are faced with many challenges. In particular many subunit antigens exhibit low immunogenicity when delivered orally [12]. As with injectable vaccines the most common strategy employed to overcome this poor immunogenicity is to include an adjuvant in the formulation. However, the development of oral vaccines is hampered by the lack of effective and safe adjuvants [12]. While many adjuvants have been developed and characterised for injection and some licenced for use, no licenced oral adjuvant currently exists [12]. The development of efficacious and safe oral adjuvants is also hampered by an incomplete understanding of the mechanisms which underlie mucosal immune responses, specifically those pertaining to the memory responses induced by vaccines.

The best characterised mucosal adjuvants are the heat-labile toxins CT and LT [312]. However, due to high levels of toxicity these are not safe for human use. This led to the generation of many derivatives with attenuated enzymatic activity and so reduced toxicity [146]. Mutations in the A subunit of the holotoxin sufficiently reduces the ADP ribosylating capacity of these toxins to render them safe for use while maintaining their adjuvanticity [146]. However, due to the presence of the B subunit, nasal delivery of both native LT and LT mutants caused Bell's Palsy in a small number of patients due to B subunit binding to the olfactory nerves and trafficking in the CNS [153]. This led to heat-labile toxins and their derivatives being viewed in a negative light by regulatory authorities [153]. While these findings have effectively closed off the nasal route for these adjuvants, it has been suggested that the sublingual and oral route of vaccination may be a viable option. Although they cannot be used as a mucosal adjuvant in humans in their wild type form, both CT and LT have been extensively characterized for their mucosal antigenicity and are the gold standard for mucosal

adjuvants. CT and LT have both been used to probe into mucosal immunology because of their high immunogenicity and immune modulating potential (section 1.11.1).

Elucidation of the key role of the IL-17 cytokine family across various aspects of mucosal immunity has had a considerable impact on the knowledge of the mechanisms underlying mucosal immunity to pathogens and vaccines [66, 79]. Initially IL-17 was found to be secreted by a distinct lineage of Th cells called Th17 cells, noted for their ability to secrete large amounts of IL-17A, IL-22 and their responsiveness to IL-23 [357, 358]. Although associated with tissue destruction and inflammation in several autoimmune diseases [359], Th17 cells also play an important protective role in the mucosa both during infection and vaccine-mediated immunity [66, 71]. Furthermore, Th17 cells have been implicated in the vaccine mediated protection against *S. pneumoniae* [360], *B. pertussis* [361], *H. pylori* [362, 363] and anthrax [355]. CT elicits strong Th17 responses following mucosal vaccination [354, 355]. While the contribution of Th17 cells to protective immunity against infection often occurs up to a week or two (evident as an adaptive response) after infection [364], IL-17 derived from other more "innate-like" sources can be detected within hours after infection [365]. These early IL-17 sources include γδ T cells, $CD8^+$ T cells, NK cells, NKT cells, neutrophils and activated monocytes [366]. McEntee *et al* have showed that oral administration of CT can elevate intestinal tissue concentrations of IL-17 within 6 hours of administration (Figure 5.1.3).

Here the specific role of IL-17 in the protective mucosal response to OCTC after vaccination with CT and CTB was investigated. Kiyono *et al* demonstrated that protection against OCTC induced by oral vaccination with muco-rice was dependent on SIgA [340]. pIgR is a receptor that transports IgA through the IECs into the lumen where it is secreted as SIgA [83]. This study highlighted the important role of pIgR in vaccine mediated protection against oral CT and LT. Moreover, intestinal SIgA antibodies against CTB are known correlates of OCV

efficacy in humans. Recently a paper was published by Cao *et al* showing that mice lacking IL-17R had significantly reduced total levels of faecal SIgA compared to wild type animals [356]. This reduction in SIgA secretion was explained by demonstrating reduced pIgR expression on IECs in these mice [356]. The study demonstrated that Th17 responses in the gut can enhance IgA secretion by augmenting both IgA secretion by B cells and up-regulating pIgR expression on IECs [356].

Taking these findings into account, WT and IL-17R$^{-/-}$ mice were orally vaccinated with CT and CTB to determine if vaccine-mediated IgA titres were reduced in IL-17R$^{-/-}$. CTB-specific IgA titres were significantly lower in IL-17R$^{-/-}$ mice compared to WT mice at all time points (Figure 5.5.2). Furthermore, CTB-specific IgA titres in the intestinal tissue were reduced in IL-17R$^{-/-}$ mice (Figure 5.5.3). However, this reduction of CTB-specific IgA found in both the intestinal tissue (Figure 5.5.3) and in faecal pellets (Figure 5.5.2) was not reflected in IgA responses in the systemic immune compartment (Figure 5.5.4 a). No differences were found in the CTB-specific titres of IgG subtypes (Figure 5.4.4 b-d) suggesting that the reduction of CTB-specific antibody titres in the intestine is due to a selective failure in induction of local mucosal immune responses. Finally, whether the reduction of CTB-specific IgA in the mice would affect the ability of the vaccine to provide protection against OCTC was assessed. Strikingly in contrast to WT mice, mice lacking IL-17R were not protected after oral vaccination with CTB and CT (Figure 5.5.5). This was evidenced by the increased fluid accumulation in the small intestine and particularly the ceca of mice deficient in IL-17R (Figure 5.5.5)

Taken together, these results show that there exists a specific role for IL-17 in the vaccine-mediated induction of anti-toxic mucosal immune responses. Furthermore, and in line with the findings of Cao *et al* [356], reduced faecal antigen-specific IgA was observed in IL-17R$^{-/-}$ mice after oral vaccination with an efficacious OCV formulation (Figure 5.5.2) in addition to

a reduced induction of local IgA in the intestinal tissue (Figure 5.5.3). Importantly, the data showed that IL-17R is indispensable for OCV-mediated protection against OCTC (Figure 5.5.5). While these findings may not hold true for the systemic compartment of the immune system (Figure 5.5.4), the data point to IL-17 as an important and indispensable cytokine for the vaccine-mediated protection against OCTC. Furthermore, the findings suggest that IL-17 may be used as an early indicator of an OCVs potential efficacy as IL-17 was found to be enhanced in the small intestine of mice shortly after oral administration of CT (Figure 5.1.3).

Although it is currently unclear whether or not the early production of IL-17 in the gut after oral administration of CT (Figure 5.1.3) is directly connected to the role of IL-17 in protective immunity it is clear that the cytokine plays a central role in this response. Determining the source and importance of this early IL-17 and its relationship to the establishment of later protective immune responses could provide a useful biomarker of protective efficacy which could aid in the development of safe and efficacious oral adjuvants which is one of the key steps towards addressing the challenges associated with developing new and improving existing oral vaccines.

5.5 – Figures

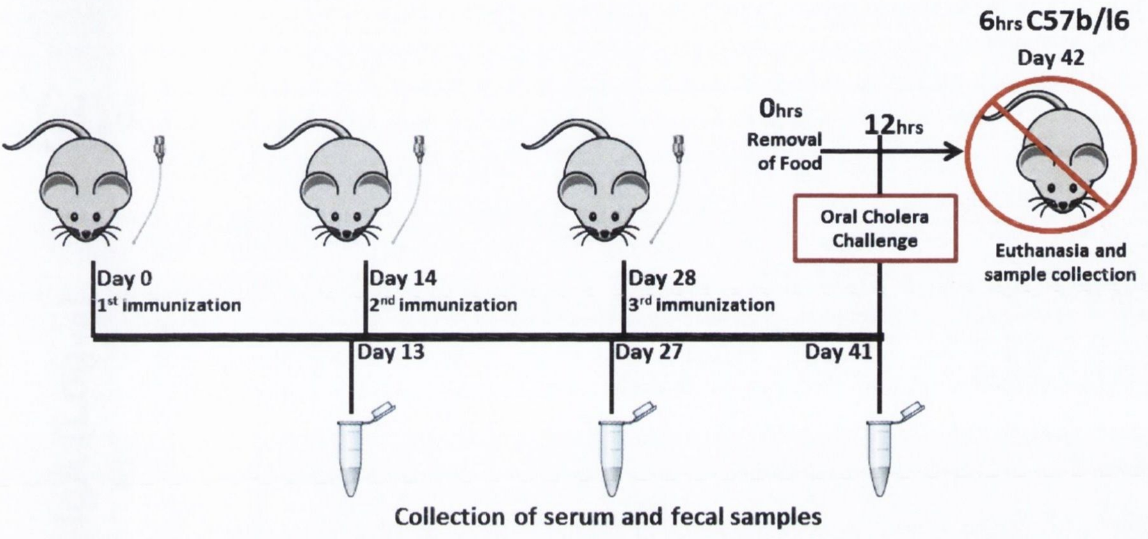


Figure 5.5.1 – Experimental timeline for evaluating oral cholera vaccines. Wildtype (C57BL/6) and IL-17R$^{-/-}$ Mice were orally vaccinated for 3 rounds two weeks apart as shown above. Components of each vaccine are detailed in the respective figure legends for each experiment. Fresh faecal pellets were collected together with serum from tail bleeds on days 13, 27 and 41 for CTB-specific antibody analysis performed on processed samples as indicated by the respective figure legends. On day 42 mice were sacrificed by cervical dislocation, organs isolated and samples harvested from these as indicated in the respective figure legends. In experiments where oral cholera toxin challenges were performed, mice were starved for 12 hours on day 41 prior to challenge. Mice were challenged for 6 hours prior to cervical dislocation on day 42.

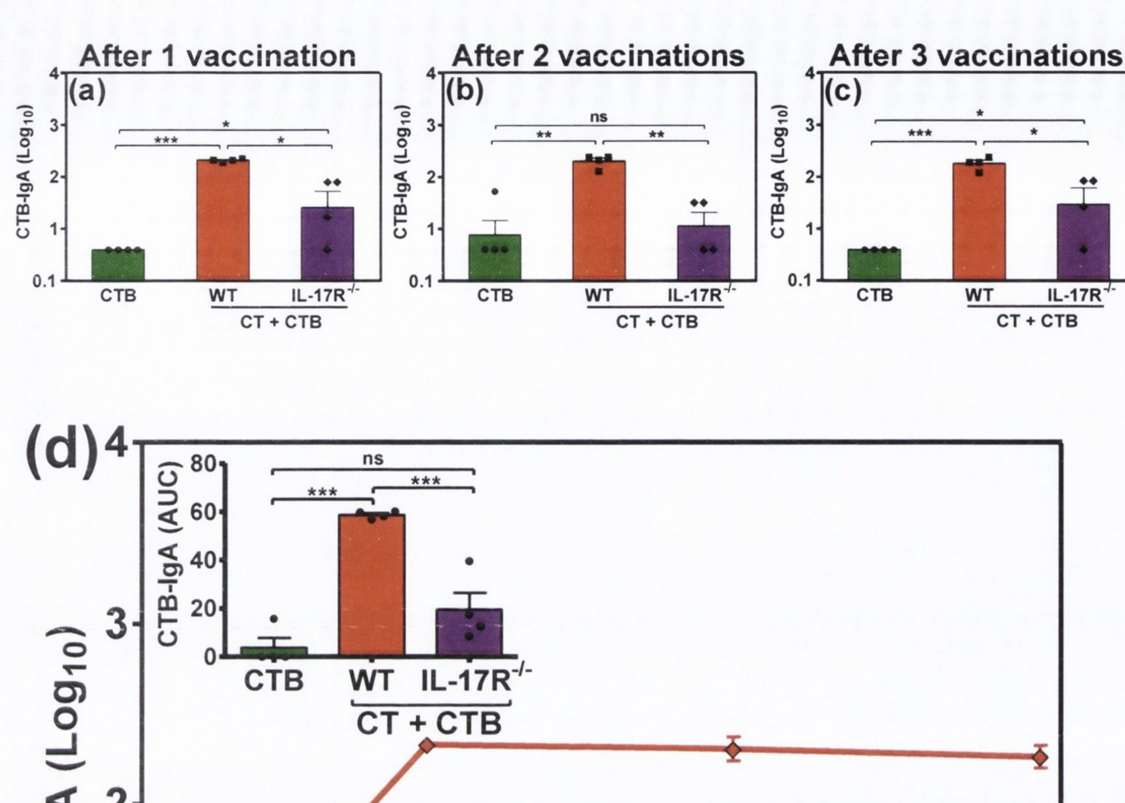


Figure 5.5.2 – IL-17R deficiency results in significantly lower antigen-specific IgA titres on faecal pellets following oral vaccination. WT or IL-17R$^{-/-}$ mice were immunised orally as per figure 5.5.1 with SBC as a control or CTB (20μg per mouse) either alone or mixed with CT (10μg). Faecal pellets were collected and supernatants recovered following centrifugation on days 13 (a), 27 (b) and 41 (c). CTB-specific IgA antibody titres in the supernatant were determined by end-point ELISA. Panels a-c present mean titres (+ SEM) for all mice per experimental group. CTB alone (CTB) versus CT + CTB (WT) versus CT + CTB (IL-17R$^{-/-}$),* $p<0.05$, ** $p<0.005$, *** $p<0.001$. Panel d presents a line graph summarizing the data. The graph superimposed in d presents the mean areas under the curve (AUC) (+ SEM) for each mouse per experimental group. CTB alone (CTB) versus CT + CTB (WT) versus CT + CTB (IL-17R$^{-/-}$), *** $p<0.001$, ns, not significant. (Representative of three independent studies)

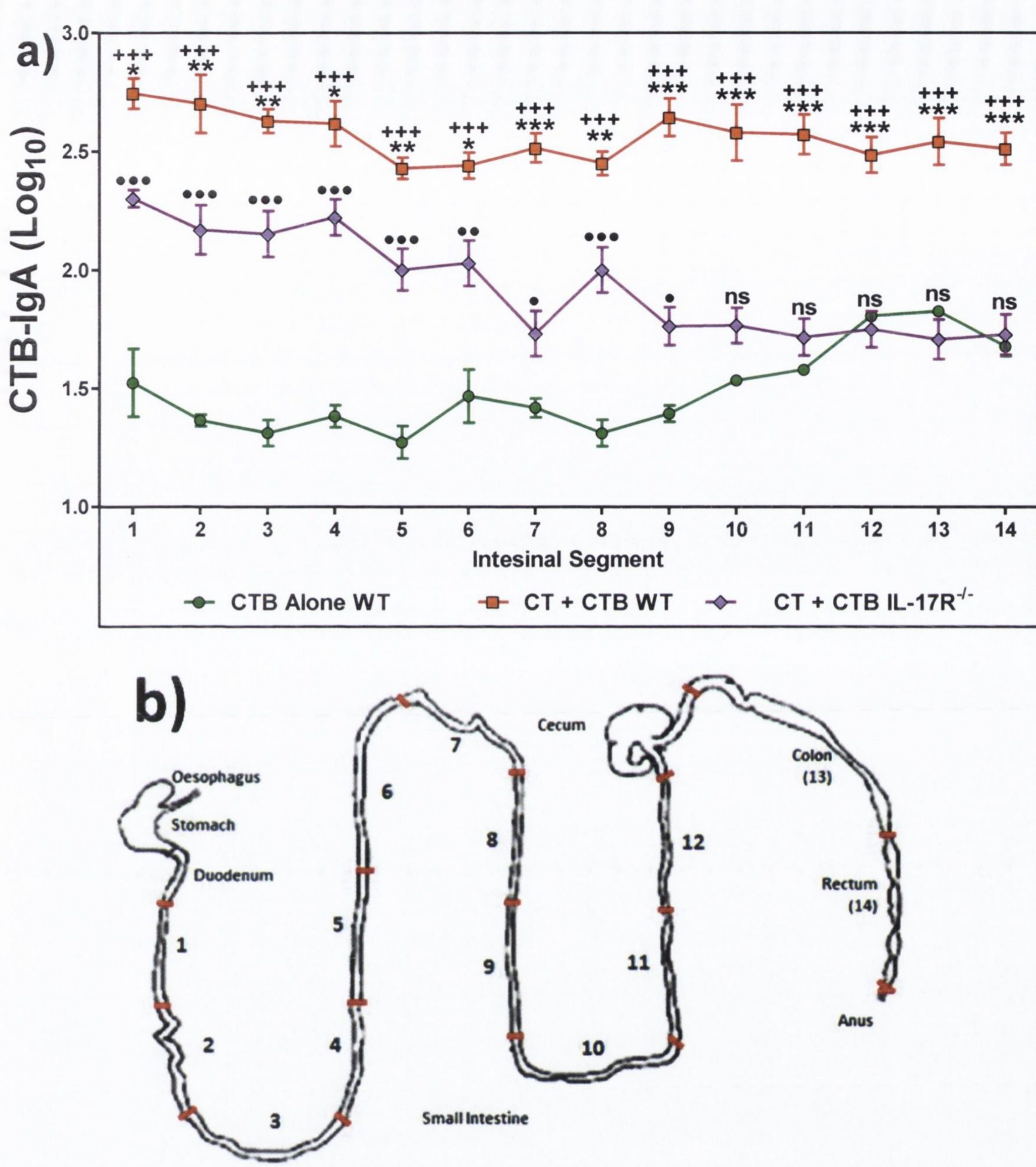


Figure 5.5.3 – IL-17R deficient mice have defective local IgA responses in the GIT following oral vaccination. WT or IL-17R$^{-/-}$ mice were immunised orally as per figure 5.5.1 with SBC as a control or CTB (20μg per mouse) either alone or mixed with CT (10μg). On day 42, mice were orally challenged with CT (20μg per mouse). After 6 hours, mice were sacrificed by CO_2 asphyxiation. 3cm segments of the small and large intestines were dissected out (b) and treated with saponin to extract the inter-tissue antibodies. CTB-specific IgA antibody titres in the supernatant were determined by end-point ELISA. Panel a presents mean titres along the length of the GIT (+ SEM) for all mice per experimental group. CTB alone (CTB) versus CT + CTB (WT), **+++** $p<0.001$. CT + CTB (WT) versus CT + CTB (IL-17R$^{-/-}$), * $p<0.05$, ** $p<0.005$, *** $p<0.001$. CTB alone (CTB) versus CT + CTB (IL-17R$^{-/-}$), ● $p<0.05$, ●● $p<0.005$, ●●● $p<0.001$, ns, not significant. (Representative of three independent studies)

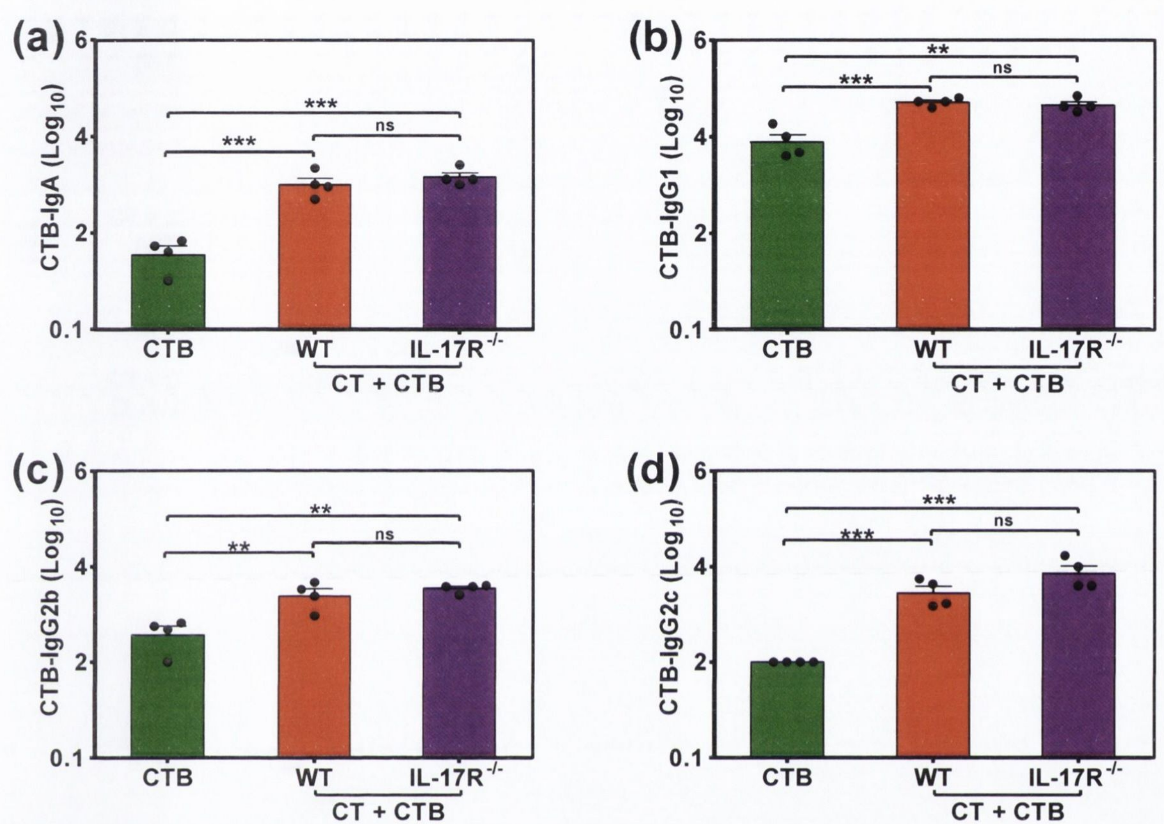


Figure 5.5.4 – There is no defect in the induction of systemic antibodies after oral immunization with CT and CTB in the absence of IL-17R. WT or IL-17R$^{-/-}$ mice were immunised orally as per figure 5.5.1 with SBC as a control or CTB (20μg per mouse) either alone or mixed with CT (10μg). Blood was obtained after collection from the tail vein and centrifugation to separate the serum. CTB-specific IgA (a), IgG1 (b), IgG2b (c) and IgG2c (d) antibody titres in the serum were determined by end-point ELISA. Panels a-d present mean titres (+ SEM) for all mice per experimental group. CTB alone (CTB) versus CT + CTB (WT) versus CT + CTB (IL-17R$^{-/-}$), ** $p<0.005$, *** $p<0.001$ ns not significant. (Representative of three independent studies)

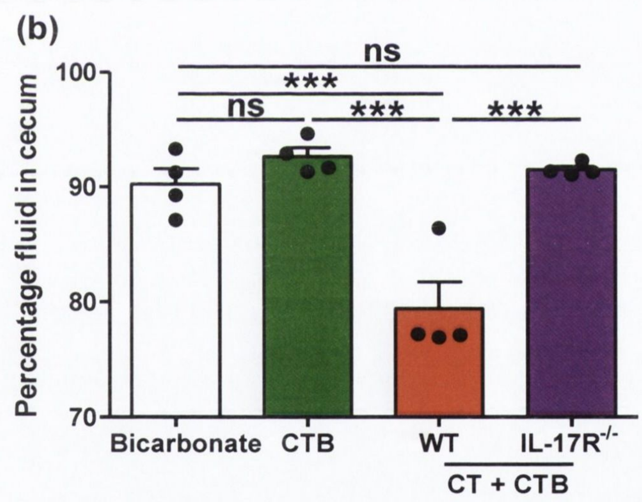


Figure 5.5.5 – Mice deficient in IL-17R are not protected against oral cholera toxin challenge after vaccination with CT and CTB. WT or IL-17R$^{-/-}$ mice were immunised orally as per figure 5.5.1 with SBC as a control or CTB (20μg per mouse) either alone or mixed with CT (10μg). On day 42, mice were orally challenged with CT (20μg per mouse). After 6 hours mice were sacrificed by CO_2 asphyxiation. The entire GIT was dissected out and photographed (a) and ceca isolated and weighed (b). Ceca were dried in an oven for 7 days at 37°C and weighed again to calculate the total percentage water lost to evaporation (b). Result b presents mean percentages (+ SEM) for all mice per experimental group. Bicarbonate versus CTB alone (CTB) versus CT + CTB (WT) versus CT + CTB (IL-17R$^{-/-}$), *** $p<0.001$, ns, not significant. (Representative of three independent studies)

Chapter Six

General Discussion

6. General Discussion

To date vaccination has been the greatest tool available to improve healthcare across the globe. It is implementable at a relatively low cost per capita, highly effective and most importantly, in principle it transcends social and economic class. From its origins in the latter half of the 17th Century by Edward Jenner, to the eradication of smallpox almost 300 years later [2], vaccination is a scientific discovery that has withstood the test of time. Louis Pasteur's concept of bacterial attenuation, Jonas Salk's development of injectable polio vaccine (IPV) and Albert Sabin's oral polio vaccine (OPV) were some of the most influential milestones in the field of vaccinology in the 20th Century. However, in terms of improving the outreach and economics of vaccines, Sabin's development of the OPV must be hailed as one of the key discoveries in terms of exploiting new routes of vaccine delivery. So effective have vaccines been that infectious diseases cause only a small number of deaths annually in developed countries relative to those cause by substance abuse and lifestyle diseases. However, the burden of infectious diseases is still enormous in the developing world, where infectious diarrhoea is a leading cause of death.

The benefits of oral vaccines are plain to see; they are cheaper to manufacture, easier to administer and so are the most amenable for implementation in mass vaccination schemes in both developing and developed countries. However, these benefits do not come free from challenges. These include the destruction of vaccine components in the acidic environment of the stomach and by enzymes in the upper portions of the gastro-intestinal tract (GIT), the dilution of vaccine formulations and the low immunogenicity of certain antigens. While solutions to all these challenges have been proposed, including delivery vehicles to protect vaccines, enhance antigen uptake and adjuvants to boost immunogenicity, none of these have been licenced for use it humans. It is noteworthy to mention that despite many attempts to

develop novel delivery systems, efficacious adjuvants and antigens the progress of improving existing and development of new oral vaccines has been sluggish at best.

The primary aim of this thesis was to develop a novel strategy to enhance the efficacy of oral vaccines. Rather than viewing the challenges outlined above as individual targets it was decided to address these in an integrated manner. Considering oral vaccination and oral drug delivery share many of the same challenges, the Single Multiple Pill (SmPill) was an ideal candidate for overcoming these. Surrounded by an enteric coat, the SmPill remains structurally intact on its passage through the stomach and upper sections of the small intestine and only begins to degrade in the more neutral pH of the more distal portions of the small intestine. Once the enteric coat is broken down it exposes a gelatin core containing many emulsion droplets and which are released when the gelatin is degraded. These properties made the SmPill a prime candidate for an oral vaccine delivery vehicle. However, early optimisation studies concluded that an adjuvant would still be required in order to enhance the immunogenicity of vaccine formulations. After an extensive search for an adjuvant McNeela *et al* (unpublished) determined that the iNKT activating glycolipid α-Galcer was a suitable mucosal adjuvant. Compared to CT, the gold standard mucosal adjuvant, α-Galcer displayed a similar ability to induce antibody responses, which are a key defensive mechanism of the mucosal immune system. Encouragingly, α-Galcer did not trigger the symptoms of diarrhoea associated with CT, and so may possess the qualities of effective oral adjuvants, namely efficacy and low toxicity.

To thoroughly evaluate both α-Galcer and the SmPill delivery system, whole cell killed (WCK) Enterotoxogenic *E.coli* (ETEC) antigen was selected. This system is composed of a killed non-toxic *E.coli* strain over-expressing colonisation factor antigen (CFA)/I referred to as formalin-killed CFA/I overexpressing *E.coli* whole cell bacteria (FK.ETEC) [310]. FK.ETEC have been extensively investigated and its immunogenicity has been demonstrated

in animal models [310, 367]. The World Health Organisation (WHO) has also recommended that the potential for increasing the antigen content and incorporating a suitable mucosal adjuvant in future candidate ETEC vaccine formulations should be evaluated and pursued [233]. Anti-CF IgA has been established as one of the key mediators of intestinal immunity to ETEC infections [151]. In all mucosal readouts of IgA production utilised, α-Galcer was comparable in efficacy to CT.

Satisfied that the adjuvanticity of α-Galcer had been sufficiently demonstrated in solution it was decided to compare the mucosal efficacy of this formulation to an identical one delivered by SmPill. It was hoped that by protecting the vaccine formulation on its transit through the stomach and by controlling its release in the intestine, it could enhance the mucosal immunogenicity of the vaccine. Faecal pellet IgA levels are often used to monitor the real-time secretion of IgA into the intestinal lumen and so is a very accurate measure of a candidate oral vaccine's efficacy. Incorporation in SmPills significantly enhanced the ability of FK.ETEC and α-Galcer to drive intestinal IgA responses. Furthermore, the induction of these responses was significantly more rapid when the vaccine was delivered by SmPill rather than in solution. Additionally, the practical benefits of the SmPill, such as pre-loading of a desired dose and ease of administration cannot be overstated. There is also an on-going concern in the development of oral vaccines that those delivered in solution may sometimes be subject to nasal and bronchiolar contamination (Dougan, G., *personal communication*) generating misleading outcomes in mouse models. Vaccine administration into the bronchiolar-associated lymphoid tissue (BALT) has been shown to lead to the generation of mucosal immune responses in the GIT [368]. Delivering vaccines by SmPill on the other hand guarantees that the intestinal immune response elicited is entirely due to the stimulation of the gut-associated lymphoid system (GALT) and so best represents the oral efficacy of that formulation.

Finally it was deemed prudent to investigate if shielding an oral vaccine from degradation was sufficient to elicit enhanced immune responses in the gut, or if the incorporation of an adjuvant is required to generate an effective SmPill vaccine delivery system. In the absence of α-Galcer, delivery of SmPills loaded with FK.ETEC did not elicit an enhancement of IgA in any readout. However, with the addition of α-Galcer, vaccination with SmPills induced potent responses against CFA/I. Enhanced immune responses were detected along the entire length of the GIT, as well as in the serum and even in the saliva. These exciting observations suggest a potential for α-Galcer adjuvanted SmPills to drive immune responses within and beyond the gut after oral vaccination.

These findings support the hypothesis that an integrated approach addressing all the challenges of oral vaccines; the need to protect labile components, target then to immune-active sites in the intestine, and to incorporate a suitable adjuvant is a viable strategy. A second generation FK.ETEC is the subject of clinical trials aiming towards developing an improved OEV [367]. Phase 1 trials without an adjuvant have been conducted in Swedish volunteers [369], and those including the dmLT adjuvant are on-going. Depending on the results of these trials, extended studies in adults, children and infants are planned for endemic regions. However, as with all clinical trials there is always a degree of uncertainty. While dmLT and FK.ETEC delivered in solution shows great promise, it is hoped that where shortfalls may occur, the integrated strategy outlined above may be utilized to address and overcome these. The strength of the pre-clinical data presented in this thesis for the enhancement of the immunogenicity of FK.ETEC by the integrated approach provides solid evidence as a proof of concept for its application in large animals which are natural hosts of ETEC (e.g. Pigs) or indeed in human subjects.

While ETEC is a major cause of diarrhoeal disease and the leading cause of Travellers' diarrhoea, Cholera is one of the most devastating diarrhoeal diseases. Contrary to ETEC

however, oral cholera vaccines (OCVs) are clinically licenced and available but these suffer from issues relating to costs and relatively poor long-term efficacy. The WHO has recommended the use of OCVs in strategies to limit the spread of cholera, which is in its 7th global pandemic [212], and evidence suggests that cases will only increase over time [282]. Furthermore, devastating outbreaks of cholera in Haiti and Zimbabwe have highlighted the need for improved OCVs which can be rapidly distributed and administered to counter epidemics [344]. To date the most effective OCV has been Dukoral®, which incorporates three killed strains of bacteria to provide O1 *V.cholerae* antigen as well as the two variants of LPS antigen, Inaba and Ogawa. The vaccine is also supplemented with recombinant CTB (rCTB) to provide protection from CT [285]. It was decided that a prototype vaccine should be developed with these basic components included, albeit with the addition of an efficacious oral adjuvant delivered in SmPills. In order to better dissect responses to such a vaccine it was decided to investigate Dukoral®'s components in isolation, namely the WCK bacterial antigens and the subunit CTB.

CTB has been shown to be a highly immunogenic peptide and other research groups have shown that delivery of this protein in high doses elicits sufficiently strong immune responses to protect mice against oral cholera toxin challenge (OCTC) [339]. However, when SmPills were loaded with increasing doses of CTB these failed to elicit sufficiently high intestinal IgA responses to protect against OCTC. While accurate loading of doses was achieved in this study it was concluded that delivering CTB alone at the doses investigated is not sufficient to provide protection. Furthermore, higher dose of CTB would be economically unfeasible, utilising current production methods, with one key goal being the reduction of the costs associated with OCVs. To address the issue of poor immunogenicity in SmPills, and the possibility of CTB degradation during SmPill manufacturing, the heat-labile adjuvant CT was incorporated into the formulation. The addition of CT generated SmPills that elicited

protective immune responses against OCTC. While the induction of faecal anti-CTB IgA was somewhat attenuated by the incorporation of CT into SmPills, this did not adversely affect the ability of this vaccine to function as effectively as its counterpart delivered in solution. This finding supported the use of SmPills as a delivery vehicle for subunit antigens and labile adjuvants.

The next stage in the development of a prototype OCV was the incorporation of WCK *V.cholerae* together with α-Galcer into SmPills. Importantly, this would determine the ability of α-Galcer to drive antibody responses against the LPS antigens present on *V. cholerae* which have been identified as an important correlate of protection [151]. Additionally, this would also determine that ability of SmPills to enhance an anti-LPS response together with α-Galcer. After demonstrating that accurate loading of WCK *V.cholerae* into SmPills is possible it was found that vaccination with these SmPills resulted in an increase in anti-LPS faecal IgA titres compared to delivery in solution. However, this study also saw a low number of mice responding to the vaccine, possibly due to an insufficient dose of antigen in both the solution and SmPill groups.

Having demonstrated the capacity of SmPills to deliver CTB and enhance the immune response against Inaba LPS it was decided to develop a prototype bivalent OCV. Lebens *et al* had successfully developed a strain of *V.cholerae* which expressed high levels of both Inaba and Ogawa LPS [307]. As this strain incorporates all 3 antigens found on the three individual strains of *V.cholerae* used in the Dukoral® preparation into a single formalin killed (FK) strain (MS1346) of *V.cholerae* while requiring only one method of inactivation, it is a much more practical and cost effective method of obtaining the antigens needed for a viable OCV [307]. FK.MS1346 *V.cholerae* was co-formulated with CTB and either α-Galcer or CT to compare the ability of both adjuvants to enhance the immune responses against the various components.

Analysis of mucosal samples from vaccinated mice in this study revealed a very interesting outcome. When mice were orally vaccinated with both CTB (a soluble antigen) and WCK bacteria (a particulate antigen) in the same formulation, α-Galcer and CT displayed differential adjuvanting capabilities for each antigen in the same mouse. CT was shown to selectively promote IgA responses against CTB, while being a poor adjuvant for stimulating anti-LPS responses. This observation is consistent with results from Abautret-Daly *et al* (unpublished) where, following oral vaccination with a WCK *H. pylori* strain α-Galcer enhances stronger faecal IgA responses while CT drives potent IL-17 responses in spleen and MLN node cells (unpublished data). Furthermore, the target bacterial-surface expressed antigens used by Abautret Daly are proteins, thus it appears not to be an issue of LPS-based antigens versus protein-based but rather that of soluble versus particulate antigens. α-Galcer in contrast to CT drives a significant enhancement of LPS-specific IgA (particulate antigen), while still maintaining an ability to drive responses against soluble antigen (CTB), albeit being somewhat less effective than CT.

However, it should be noted that in this study the number of mice responding to the WCK antigens was again lower than the response to the soluble antigen in the same mouse. This suggests that while the dose of both CTB and the adjuvant was sufficient to elicit a response from all mice in the treatment groups, the dose of FK.MS1346 may need to be increased in subsequent studies to increase the number of mice responding. This would also be a further step to evaluating the hypothesis that CT may be a poor driver of antigen specific IgA responses against particulate antigens.

Since that no significant differences in CTB-specific IgA titres were detected between mice vaccinated with either α-Galcer or CT after three rounds of oral immunisation it was decided to investigate if these titres would correlate to protection against OCTC. Mice vaccinated with

CT and CTB were rendered immune from OCTC while this protection was not as clearly evident in the mice vaccinated with CTB and α-Galcer.

The OCV prototype α-Galcer adjuvant formulation encapsulated in SmPills was compared to the currently licenced clinical OCV Dukoral® in terms of immunogenicity and protective efficacy. Faecal antigen-specific IgA results indicated that the prototype OCV in SmPills elicited not only higher titres but also a more rapid induction than Dukoral®. While intestinal tissue antibody titres were similar between groups, the elevation of faecal titres, a "real-time" measure of the production and translocation of antibodies into the intestinal lumen is very encouraging. Furthermore, consistent with the findings from the first results chapter, α-Galcer is required for the ability of SmPills to drive faecal antibody responses against cholera antigens similarly to the case with ETEC antigens. However, it was also observed as in previous studies with WCK *V.cholerae* that the current dose of bacteria is likely not sufficient to elicit immune responses from all mice in the group and therefore will need to be increased to a point where all mice respond. Finally an OCTC was conducted to determine if Dukoral® or the SmPill prototype OCV could provide a protective anti-toxic response. Neither vaccine however, provided sufficient protection from OCTC in this study.

This chapter demonstrated that α-Galcer is a versatile mucosal adjuvant capable of enhancing the immune response against a variety of antigens. Furthermore, SmPills are also capable of enhancing this response when the antigens and adjuvants are incorporated. However, it is clear that SmPills do not offer a "one size fits all" approach to oral vaccination and that each formulation will need to be carefully optimised. The oil phase used to incorporate antigens and adjuvants into SmPills may need to be specifically tailored to different formulations to ensure optimal uptake of these components into SmPills and most importantly to preserve their antigenicity and adjuvanticity.

The final chapter of this thesis sought to investigate the mechanisms that underlie the oral vaccine mediated protection against OCTC. The prototype OCV tested at the end of the previous chapter failed to elicit satisfactory protection either in solution or in SmPill despite enhancing faecal anti-CTB IgA titres to similar levels as CT. This observation begged the question, are there other mechanisms beside IgA that play a decisive role in the vaccine-mediated protection against OCTC? One result from this study in particular stood out and may hold an explanation as to α-Galcer's poor protective efficacy. *Ex vivo* re-stimulation of both splenocytes and mesenteric lymph node (MLN) cells with MS1346 *V.cholerae* demonstrated that cells from CT vaccinated mice produced significantly higher amounts of antigen-specific IL-17A compared to mice vaccinated with α-Galcer. These findings are consistent with observations by Abautret-Daly *et al* in our laboratory who similarly found α-Galcer to be a poor driver of antigen-specific Th17-like responses by re-stimulated lymphocytes (unpublished data). Considering that the most striking difference found between the antigen-specific immune responses elicited by CT and α-Galcer adjuvanted formulations was related to antigen-specific IL-17, the role of this cytokine in the vaccine-mediated protection against OCTC was investigated.

Preliminarily studies in our laboratory conducted by McEntee *et al* revealed that CT induced high amounts of IL-17A production after oral administration in naïve mice (unpublished data). McNeela *et al* also found that in comparison to TLR agonists, CT drives much stronger T cell proliferation and IL-17A production after oral vaccination (unpublished data). Other groups have also shown that CT is capable of promoting Th17 responses [354, 355] and have illustrated the importance of Th17 cells in mucosal immune responses and homeostatis [66, 79].

Despite this, there is no evidence currently available linking CT's ability to drive Th17-like responses and protection against OCTC. A paper published by Tokuhara *et al* determined that

oral vaccine-mediated protection against OCTC was dependent on SIgA, specifically related to the presence of the polymeric Ig receptor (pIgR), which is a key receptor in the transport of IgA through intestinal epithelial cells (IECs) from the lamina propria into the lumen of the gut [340]. Recently, a paper was published by Cao *et al*, showing that concentrations of SIgA in the lumen of IL-17R$^{-/-}$ mice were significantly lower than in wild type mice [356]. The group showed that this defect in SIgA secretion was linked to reduced basal pIgR expression on IECs [356]. However, the study only investigated the impact of reduced pIgR expression in terms of intestinal homeostatis and not whether this would impair a vaccine-mediated mucosal immune response.

Following vaccination with CT and CTB it was found that IL-17R$^{-/-}$ mice had significantly lower CTB-specific IgA titres in faecal pellet samples compared to wild type (WT) mice, an effect that was not reflected in terms of CTB-specific serum IgA or IgG. While these findings definitively demonstrated a mucosal-specific role for the IL-17R in the vaccine-mediated enhancement of antigen-specific IgA responses in the gut, it still remained to be demonstrated whether this defect would render IL-17R$^{-/-}$ mice susceptible to OCTC following oral vaccination with an efficacious formulation. IL-17R$^{-/-}$ mice vaccinated with CT and CTB were not protected from OCTC while WT mice were a result, which directly implicates the IL-17R in the protective vaccine-mediated response to OCTC.

It is evident that encapsulation in SmPills and co-formulation with α-Galcer proved to be an excellent strategy for improving the oral efficacy of a novel OEV however; this approach may not be directly transferrable to other enteric infections and antigens. Clearly the formulation used to enhance the immunogenicity of FK.ETEC is not directly applicable to the prototype OCV. While the benefits of incorporating α-Galcer are clear, further optimisation of the oil-phase used to load antigens and adjuvants into SmPills may be required. Additionally, the duality of adjuvanticity of both α-Galcer and CT may suggest that although α-Galcer is an

excellent adjuvant for particulate antigens, it may not possess the ability to drive the immune mechanisms that govern protection against OCTC.

The data suggest that faecal IgA titres may not be the sole factor that serves a protective function against OCTC and from the data at hand one could speculate that IL-17 may play an additional role in IgA-mediated protective immunity. Therefore in order to improve the protective efficacy of α-Galcer it may be necessary to consider the addition of an IL-17 driving adjuvant such as an LT mutant to the current formulation. This might endow the current formulation with the ability to provide enhanced protection against OCTC by a combined mechanism of enhanced IgA production via α-Galcer and the engagement of other mechanisms which contribute to protection against OCTC by an IL-17 driving adjuvant.

Further study of the definitive role of IL-17 and the IL-17R in protection against enteric pathogens and in vaccine mediated mucosal immunity is clearly warranted to identify and exploit the exact mechanism in order to improve the efficacy of oral vaccines.

Overall the findings of this thesis support the hypothesis that an integrated approach to the development of non-living oral vaccines, protecting labile antigens, delivering them to the optimal location in the GIT and incorporating a suitable mucosal adjuvant can greatly enhance immunogenicity. Hopefully these finding will lead to the development of new and improvement of existing oral vaccines against enteric infections, and continue in improve world health one pill at a time.

Chapter Seven

References

7. References

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