Novel approaches to mucosal vaccine development

Strategies in vaccine antigen production, construction of a novel mucosal adjuvant and studies of its mode of action

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Cover illustration: Figure adapted by Manuela Terrinoni from the Garden of the Earthly Delights by Hieronymus Bosch. Hell and Heaven represents how the world would be imagined without or with vaccination and ideally with the tools produced in this thesis. Juxtaposing these two imaginary worlds on a scale, the balance is shifted heavenwards, since vaccination has a heavier and deeper impact in terms of the human condition and the world we share.

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Consider your origins:
you were not made to live as brutes,
but to follow virtue and knowledge.

Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e conoscenza.

Dante Alighieri

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ABSTRACT

Although most infections begin at a mucosal surface and may be prevented by effective vaccine stimulation of the local mucosal immune system, there are so far only a few mucosal vaccines available for human use. This thesis spans several areas that are important for future development of mucosal vaccines.

Future vaccine development will depend in part on the efficient production of recombinant antigens produced in bacterial expression systems. To avoid current problems with the use of antibiotics to maintain expression plasmids, an *E. coli* strain capable of producing recombinant proteins using vectors maintained without the need antibiotic was generated. The method is based on deletion of the essential *lgt* gene encoding a (pro)lipoprotein glyceryl transferase and complementing it with an expression vector carrying the non-homologous *lgt* gene from *V. cholerae*. A similar *V. cholerae lgt*-deleted strain was also constructed using the *E. coli lgt* gene for complementation. The strains had similar growth and production characteristics as their wild-type counterparts but maintained their expression plasmids without the need for antibiotics. The system was used to express two recombinant vaccine proteins, cholera toxin B subunit and a fusion protein for vaccination against atherosclerosis.

In the development of mucosal vaccines, it is often important to enhance immune responses using adjuvants, since most mucosally administered antigens are poorly immunogenic. Cholera toxin (CT) is the most powerful mucosal adjuvant known but is too toxic for human use. A mutated CT derivative (mmCT) was constructed and expressed in an engineered strain of *V. cholerae*. mmCT induced 1000 times less cAMP than native CT in a mouse thymocyte toxicity assay, was non-toxic in an infant mouse model and yet retained similar adjuvant properties as native CT. We suggest that mmCT is a promising candidate for use in future mucosal vaccines.

The mode of adjuvant action of mmCT and native CT was investigated using human and mouse antigen-presenting cells, which are primary target cells for adjuvants. Both molecules were found to activate cyclic AMP/protein kinase A-dependent canonical NF-κB signaling associated with inflammasome activation. The activation of these pathways was found to induce expression of two immunomodulatory proteins, THSB1 and ITGB1, as well as increased expression and activation of IL-1β, a cytokine which has been shown to play an important role for the adjuvant action of CT and mmCT.

Keywords: vaccine development, plasmid maintenance, Gram-negative bacteria, essential genes, complementation, Cholera Toxin, adjuvanticity, NF-κB, mmCT

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SAMMANFATTNING PÅ SVENSKA

Trots att de flesta infektioner sker på eller startar vid en slemhinneyta och skulle kunna förhindras genom effektiv vaccinstimulering av slemhinnans lokala immunsystem finns det än så länge bara ett fåtal slemhinnevacciner framtagna. Avhandlingen spänner över flera områden som är viktiga för framtida utveckling av mukosala vacciner, från en ny metod för produktion av rekombinanta vaccinproteiner till utveckling av ett nytt icke-toxiskt men ändå potent slemhinneadjuvans och studier av mekanismerna för dess adjuvansverkan.

Ett viktigt område inom vaccinutveckling gäller effektiv produktion av rekombinanta antigener som produceras i bakterier. Sådan produktion baseras idag huvudsakligen på s.k. expressionsplasmider som bibehålls i produktionsbakterien genom selektion med antibiotika. Med det ökande problemet med antibiotikaresistens avrådes idag starkt från användning av antibiotika i sådana processer. För att lösa detta problem har vi tagit fram och karakteriserat en *Escherichia coli*-stam som kan producera rekombinanta proteiner med användning av plasmidvektorer som bibehålls utan behov av en antibiotikaresistensgen. Metoden baseras på borttagandet av en väsentlig gen från bakteriens kromosom, *lgt*-genen som kodar för ett (pro) lipoprotein-glyceryltransferas i *E. coli*-värdstammen och ersättning av förlusten genom låta expressionsvektorn innehålla en motsvarande men icke-homolog *lgt*-gen från *Vibrio cholerae*. Samma tillvägagångssätt användes också för att generera en *lgt*-deleterad *V. cho*lerae-stam med användning av *lgt*-genen från *E. coli* för komplementering. De genererade stammarna uppvisade liknande tillväxt- och produktionsegenskaper som deras motsvarande vildtyp-stammar men bibehöll sina plasmider med extremt hög stabilitet utan behov av antibiotika. Det nya systemet har framgångsrikt använts för att uttrycka två rekombinanta vaccinproteiner, ett lösligt protein (koleratoxinets B-subenhet) och ett som bildar olösliga inklusionskroppar (ett fusionsprotein för vaccination mot åderförkalkning).

Vid utveckling av slemhinnevacciner är det ofta viktigt att kunna förstärka immunsvaret med hjälp av adjuvans, eftersom de flesta antigener när de administreras på slemhinnor är dåligt immunogena. Det starkaste kända slemhinneadjuvanset är koleratoxin (CT), som dock är alldeles för toxiskt för att användas på människor. Vi har därför konstruerat ett muterat CT-protein (mmCT) som vi kunnat producera och rena från en genetiskt konstruerad stam av *V. cholerae*. Vi kunde visa att mmCT-proteinet inducerade 1000 gånger mindre cykliskt AMP än nativt CT i ett cellsystem och helt saknade toxicitet i en musmodell men ändå bibehöll liknande adjuvansegenskaper som nativt CT. Vi ser därför mmCT som ett lovande adjuvans för användning i framtida slemhinnevacciner.

I avhandlingen studerades också mekanismerna för adjuvansfunktionen hos både mmCT och CT genom att studera deras effekter på antigenpresenterande celler (APC) från både mus och människa som är de primära målcellerna för dessa och andra adjuvansmolekyler. Bägge proteinerna visades inducera aktivering av cykliskt AMP/proteinkinas A-beroende s.k. kanonisk (klassisk) NF-kappaB signalering associerad med aktivering av cellernas s.k. inflammasomsystem. Aktiveringen av dessa signalsystem visade sig inducera uttryck av två till varandra knutna immunmodulerande proteiner, THSB1 och ITGB1, jämte såväl ökad expression som förstärkt aktivering av IL-1beta, ett cytokin som har visat sig spela en viktig roll för både mmCT:s som CT:s adjuvansverkan.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Manuela Terrinoni, Stefan L. Nordqvist, Susanne Källgård, Jan Holmgren, Michael Lebens

A novel non antibiotic, lgt-Based selection system for stable maintenance of expression vectors in Escherichia coli and Vibrio cholerae.

Applied Environmental Microbiology 2017 84:e02143-17. https://doi.org/10.1128/AEM.02143-17.

II. Michael Lebens, Manuela Terrinoni, Stefan L. Karlsson, Maximilian Larena, Tobias Gustafsson-Hedberg, Susanne Källgård, Erik Nygren, Jan Holmgren.

Construction and preclinical evaluation of mmCT, a novel mutant cholera toxin adjuvant that can be efficiently produced in genetically manipulated Vibrio cholerae

Vaccine 2016 34, 2121–2128, https://doi.org/10.1016/j.vaccine.2016.03.002

III. Manuela Terrinoni, Jan Holmgren, Michael Lebens and Maximilian Larena

Requirement for cyclic AMP/protein kinase A-dependent canonical NFκB signaling in the adjuvant action of Cholera Toxin and its non-toxic derivative mmCT

Frontiers in Immunology 2019 10:269. doi: 10.3389/fimmu.2019.00269

IV. Manuela Terrinoni, Jan Holmgren, Michael Lebens and Maximilian Larena

Proteomic analysis of cholera toxin adjuvant-stimulated human monocytes identifies Thrombospondin-1 and Integrin-β1 as strongly upregulated molecules involved in adjuvant activity

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PAPER NOT INCLUDED IN THE THESIS

Maximilian Larena, Jan Holmgren, Michael Lebens, Manuela Terrinoni and Anna Lundgren

Cholera toxin, and the related nontoxic adjuvants mmCT and dmLT, promote human Th17 responses via cyclic AMP-protein kinase A and inflammasome-dependent IL-1 signaling.

Journal of Immunol, 2015. 194(8): p. 3829-39.

CONTENT

ABBREVIATIONS V

1 INTRODUCTION 1

GENERAL OVERVIEW 1

VACCINE DEVELOPMENT: ANTIBIOTIC FREE ANTIGENS PRODUCTION APPROACHES 3

VACCINES 4

History of vaccines 4

History of mucosal vaccines 4

Mode of action of vaccines: induction of adaptive immunity and immunological memory 5

Protective immunity at mucosal surfaces 7

Types of Vaccines 9

Mucosal vaccines 11

Remaining challenges 12

Specific challenges for mucosal vaccines 13

ADJUVANTS – A TOOL TO OVERCOME MANY VACCINE BARRIERS 15

Definition and mode of action 15

Mucosal adjuvants 18

Cholera toxin as model for mucosal adjuvants 19

Next generation CT- derived mucosal adjuvants 20

Others 22

2 AIMS 23

3 METHODS 25

4 RESULTS AND DISCUSSION 37

5 GENERAL DISCUSSION 59

ACKNOWLEDGEMENTS 65

REFERENCES 67

ABBREVIATIONS

Ag	Antigen
AC	Adenylate cyclase
APC	Antigen-presenting cell
ADP	Adenosine diphosphate
ALOX-5	Arachidonate 5-Lipoxygenase
BCG	Bacillus Calmette-Guérin
BM	Bone marrow
cAMP	Cyclic adenosine monophosphate
CAPE	Caffeic acid phenethyl ester
CT	Cholera toxin
CTA	Cholera toxin A subunit
CTB	Cholera toxin B subunit
CFTR	Cystic fibrosis transmembrane conductance regulator
CpG	Cytosine-phosphate guanine
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
ER	Endoplasmic reticulum
ERAD	Endoplasmic Reticulum-associated degradation
ETEC	Enterotoxigenic *Escherichia coli*
GALT	Gut-associated lymphoid tissue
GC	Germinal center
GTP	Guanosine triphosphate
GM1	Galactosyl - N –acetylgalactosaminyl - (N-acetylneuraminyl) - galactosyl-glucosyl-ceramide
H-89	Dihydrochloride
HA	Hemagglutinin
HIV	Human immunodeficiency virus
HLA-DR	Human Leukocyte Antigen – DR isotype
HPV	Human papilloma virus
i.n.	Intranasal
ITGB1	Integrin beta-1
i.v.	Intravenous
IKK	Inhibitor of Kappa B Kinase
LPS	Lipopolysaccharide
LT	*Escherichia coli* labile toxin
MALT	Mucosal-associated lymphoid tissue
MHC	Major histocompatibility complex

MPL	Monophosphoryl lipid A
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor-kappa B
NIK	Nuclear factor-kappa B inducing kinase
NK	Natural killer cell
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
p.o.	Peroral
Poly (I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptors
QS	Quillaja saponaria
SEB	Staphylococcal enterotoxin B
sIgA	Secretory immunoglobulin A
s.l.	Sublingual
Tfh	T follicular helper cell
Th	T helper cell
TNF	Tumor necrosis factor
TLR	Toll-like receptor
Treg	Regulatory T cell
THSB1	Thrombospondin-1
VLP	Virus-like particle

1 INTRODUCTION

GENERAL OVERVIEW

More than 200 years have passed since Edward Jenner introduced the idea of disease prevention by vaccination [1]. Now vaccines are considered fundamental to the control of many infectious diseases. To date, vaccination programs have led to the global eradication of smallpox, the almost global elimination of polio, diphtheria, diseases caused by *Haemophilus influenzae*, meningitis A and C, and several strains of pneumococci [2]. Moreover, the role of vaccines in infectious disease control is now even more important with the rapid increase of antimicrobial resistance, endangering the efficacy of antibiotics. This is because vaccination can prevent infections, therefore decreasing the use of antibiotics and reducing microbial resistance development [3].

A large majority of licenced vaccines are either currently composed of whole bacteria or viruses, that have either been inactivated (killed vaccines), or have been attenuated to not cause disease (live attenuated vaccines). In modern vaccine development, however, there is an increasing trend to instead try to use purified vaccine antigens as subunit vaccines. Many of these subunit vaccines are made up of recombinant proteins that are produced on plasmid expression systems, which are maintained in host bacteria using selection based on antibiotic resistance. The problem is that regulatory pharmaceutical agencies are increasingly limiting the use of antibiotics, in order to minimize the propagation of antibiotic resistance genes and reduce the risk of antibiotic contamination of products [4].

Consequently, part of this thesis describes a practical approach to eliminate the use of antibiotics in plasmid maintenance based on deletion of an essential chromosomal gene in the host strain and the use of a complementing gene carried on the expression vector. This system is based on the deletion of the essential chromosomal *lgt* gene in a host *Escherichia coli* or *Vibrio cholerae* strain, and complementing it using an expression vector carrying a corresponding but non-homologous *lgt* gene from *V. cholerae* or *E. coli*. The generated strains had similar growth and production characteristics to their

wild-type counterparts but maintained their plasmids with extremely high stability without the need for antibiotics.

Most infections start at mucosal surfaces primarily because the gastrointestinal, genitourinary, and respiratory tracts represent a huge interface of more than 400 square meters with the environment. It is thus reasonable to propose that eliciting protective immune responses at these mucosal sites should be an aim of vaccination against many pathogens and that vaccines administered topically at the mucosal sites are in general the most appropriate means of achieving protective immunity [5].

Despite the numerous potential advantages of mucosal administration only a few mucosal vaccines are available. These are orally or nasally administered vaccines against diseases such as cholera, polio, rotavirus diarrhoea, typhoid fever, and influenza. All of these licenced mucosal vaccine are whole live attenuated, or inactivated bacteria or viruses with inherent adjuvant activity [5-7]. This is because purified protein antigens are in general poorly immunogenic when delivered orally, and mucosal protein or other subunit vaccines usually need to be co-administered with an adjuvant that increases their immunogenicity and prevents the induction of "oral tolerance" [8].

Some of the most promising mucosal adjuvants to date are based on the enterotoxins of *V. cholerae* (cholera toxin, CT) or *E. coli* (heat-labile enterotoxin, LT). CT and LT are the most potent mucosal adjuvants known to date, but their high toxicity precludes their use in humans [9, 10]. Consequently, in the second part of this thesis a CT-derived mucosal adjuvant mmCT has been developed, which is essentially non-toxic whilst retaining much of the adjuvant activity of native CT, and also been able to define in considerable detail some aspects of the mode of adjuvant action of both the native and mutant molecules.

VACCINE DEVELOPMENT: ANTIBIOTIC FREE ANTIGENS PRODUCTION APPROACHES

In the development of systems for the industrial production of recombinant proteins, protein expression genes are usually placed on expression plasmids, allowing the bacteria to survive in presence of antibiotic in the culture media. Antibiotics only allow the bacteria carrying the expression plasmid to survive, therefore reducing the chance of bio-contamination in production processes. However, the use of antibiotics is undesirable for several reasons. From the point of view of production efficiency, the expression of antibiotic resistance genes imposes a metabolic burden on the cells, resulting in reduced growth rates and lower cell densities. Also, the final products may be contaminated with antibiotic residues leading to increased costs for purification and quality control. Finally, there are the dual dangers of contamination of the environment with antibiotic residues and the horizontal gene transfer of antibiotic resistance genes if the DNA is released into the environment, both of which can contribute to emergence of antibiotic-resistant pathogens [4, 11-13].

Due to these considerations, alternative strategies for the maintenance of plasmids without the need for antibiotics have been devised. The most commonly used are: 1) Auxotrophic complementation [11]; 2) Post segregational killing exploiting naturally occurring plasmid maintenance systems [14]; and 3) Operator repressor titration in which the non-expressed *lac* operator sequence functions as the vector-borne selection marker [15].
A final approach is to delete an essential chromosomal gene and to use the expression vector to complement it [16]. This has two main advantages. It confers extremely high stability to the plasmids since the cells cannot survive without the complementing plasmid and secondly, the cells can be cultured in any growth medium in which the parental strain can grow and do not require any special additives. However, due to the need for the deleted gene for cell survival, a convenient means of maintaining the parental strain and transforming the strain with expression vectors can be problematic. In Paper I we have devised a method in which an essential gene required under all growth conditions was deleted from the chromosomes of both *E. coli* and *V. cholerae* and in each case complemented by a non-homologous gene with the same function. This system allowed us to produce recombinant vaccine proteins in two different host strain species.

VACCINES

HISTORY OF VACCINES

As early as 1000 CE in China, Turkey and India, smallpox inoculation (or variolation) was employed to provide protective immunity. But only in the 18th century was the practice introduced on a large scale. The systematic introduction of mass smallpox immunization and a coordinated global effort culminated in its global eradication in 1979 [17]. In the latter part of the 19th century Louis Pasteur found that he could attenuate pathogenic bacteria by exposure to adverse conditions [18]. Work focused on the pragmatic inactivation of whole bacteria for use in vaccines, even though it was not yet known how they were working. During this period inactivated whole-cell vaccines against rabies, anthrax, typhoid, cholera, and plague, were produced and tested with mixed results. Later, methods for growing viruses in the laboratory led to rapid discoveries and innovations, including the creation of vaccines for polio [19]. Other common childhood diseases such as measles, mumps, and rubella were also targeted and vaccines for these diseases have reduced the disease burden dramatically [2]. The explosive growth in our understanding of the immune system and the nature of protective immunity in recent years has led an expansion of the range of diseases that can potentially be treated by vaccination. Rational approaches aimed at harnessing the immune system to the best effect have replaced the early era of trial and error.

HISTORY OF MUCOSAL VACCINES

In its crudest form, mucosal immunity can be traced back to the second century BC during the rule of the despotic king Mithridates VI-Euptor. He routinely ingested the blood of ducks that had been fed a formula of poisonous weeds in an effort to elicit resistance to a commonly used plant-derived poison [20].

In the latter half of the 19th century, important attempts to protect against serious disease were made. Oral immunizations with bacteria such as *V. cholerae, S. dysenteriae, M. tuberculosis, Yersinia multocida, Y. pestis and Corynebacterium diphtheriae* were undertaken with varying degrees of success. However, while serum antibodies were found to be induced by oral immunization, there was a degree of scepticism as to whether they could be considered as markers of protection. It was not till the early 1890s that the

potential of mucosal immunization was discovered when Besredka described antibodies in external secretions (gut mucosal antibodies). This led to several large (but not rigorously controlled) studies in military staff in India and elsewhere to evaluate the efficacy of oral killed whole-cell vaccines against cholera and shigellosis, usually with good reported results [20]. Calmette and Guérin developed an attenuated variant of *Mycobacterium bovis*, Bacille Calmette-Guérin (BCG) which was first tested in infants as an oral vaccine. It was soon changed to be given intradermally however, due to adverse effects [21].

Thus, considering the early history of vaccine development, it is perhaps surprising that there are only a few mucosal vaccines available compared with more than 30 licensed parenteral vaccines.

MODE OF ACTION OF VACCINES: INDUCTION OF ADAPTIVE IMMUNITY AND IMMUNOLOGICAL MEMORY

Vaccines are designed to elicit immunity against a specific pathogen based on a rapid protective immune response. This is achieved by exploiting adaptive immunity characterized by specific recognition of pathogens and long-lasting memory. In currently available vaccines, the main effectors are antibodies produced by B cells, which specifically bind a toxin or pathogen leading to its inactivation and or removal. However, the role of T cell responses is of equal importance, since they provide essential help in the induction of high affinity antibody and memory B cells and effector T cells may also contribute directly to protection in many vaccines.

The magnitude, quality and duration of adaptive immune responses to infections or vaccines are greatly influenced by the innate immune system. The innate immune system has limited specificity and lacks memory, but it is the first to respond to a potential infection in ways promoting the activation of the adaptive immune system. These components of the innate immune system recognize PAMPs (pathogen-associated molecular patterns) in a non-specific manner using PRRs (pattern-recognition receptors). Activation of the innate immune system also attracts cells involved in initiating an adaptive response. Principal amongst these are antigen presenting cells (APCs) that, once activated, process and present the antigen via major histocompatibility

complex (MHC) class molecules together with co-stimulation signals (CD80/CD86) to T cells [22].

Cooperation between APCs and T-lymphocytes is required for the initiation of adaptive immunity. $CD4^+$ T cells recognize antigenic peptides displayed by class II MHC molecules via specific receptors on their cell surface and when activated, provide signals (CD40L, cytokines etc.) which result in their own further activation and differentiation into specialized T cells (Tregs, Th1, Th2, Tfh, Th17) with distinct functions. They can thereby promote clearance of extracellular and intracellular pathogens both directly and through the activation of specific B cells and $CD8^+$ T cell populations. $CD8^+$ T cells are important mainly in the control and clearance of intracellular pathogens following activation through presentation of antigens in the context of MHC class I [23-26].

Whilst most (>90%) effector T cells die within a few days, some of the progeny that proliferated in response to the vaccine antigen, develop into long-lived memory T cells which may persist lifelong, even in the absence of antigen exposure. Some are resident within specific organs such as the intestine, the lungs, and the skin. Indeed, mucosal memory T cells are central for protection against mucosal infections and novel vaccine strategies against viral (influenza, respiratory syncytial virus [RSV]) or bacterial (pertussis) mucosal pathogens have as main goal their induction or maintenance [27-30].

The adaptive B cell response is characterized by the parallel development of plasma cells producing antibodies and often very long-lived B memory cells. Protective antibodies mainly recognize antigenic epitopes on the surface of extracellular pathogens or toxins. These antibodies can then exert their protective function in various ways, e.g. by promoting phagocytosis or complement mediated lysis of the pathogen or by neutralizing viruses and toxins preventing their attachment to target cell receptors. When exposed to antigen, B-cells are activated in two ways, one being T cell-dependent taking help from helper T cells (Th cells) and the other being T-cell independent. The latter is activated mainly in response to bacterial (*Streptococcus pneumoniae, Neisseria meningitidis, Haemophilus influenzae, Salmonella typhi*) capsular polysaccharides (PS), where B cells are activated, proliferate, and differentiate into plasma cells without undergoing affinity maturation in germinal centers.

The T cell-dependent route of B cell activation is primarily directed against protein antigens. Activated B cells upon T cell interaction undergo a germinal center (GC) reaction characterized by clonal proliferation, isotype switching and affinity maturation. In the GC reaction, long-lived plasma cells and memory B cells also are generated [31-33].

Long-lived plasma cells migrate toward the bone marrow (BM) from where they can produce antibodies for extended periods. In such BM niches, plasma cell survival and antibody production may persist for years. The duration of antibody responses reflects the number and/or quality of long-lived plasma cells generated by an infection or immunization [34-36].

Memory B cells (as opposed to long-lived plasma cells) can also be extremely long-lived (Functional antigen-specific B-cell memory has been demonstrated for longer than 10 years after primary oral cholera vaccination [37]). They exist as circulating resting cells until they re-encounter a specific antigen. They then proliferate differentiating into plasma cells secreting large amounts of high-affinity antibodies that can be detected in the serum within a few days after exposure to a previously encountered pathogen or a booster immunization. Antigen-specific B memory cells following immunization are present in much larger numbers than naïve B cells recognizing the same antigen. Since the affinity of surface Ig from memory B cells is also increased after the first response, the activation of B memory cells is easier as compared to the naïve B cells; thus, memory B cells can be activated by lower amounts of antigen and without $CD4^+$ T-cell help, although T-cell help supports a new round of GC responses, further magnifying the levels of high affinity antibodies (and additional memory cells) [38].

PROTECTIVE IMMUNITY AT MUCOSAL SURFACES

Mucosally administered antigens are taken up from the lumen of the digestive or respiratory tract by so-called M cells located in the follicle-associated epithelium (FAE) of organized lymphoid mucosal structures known as MALT (mucosal-associated lymphoid tissue). MALT is the inductive site where mucosal immune responses are initiated. It includes the gut-associated lymphoid tissue (GALT) which comprises the Peyer´s Patches and isolated lymphoid follicles of the small intestine, the colon patches and the appendix; the nasopharyngeal-associated lymphoid tissues (NALT) and the bronchial-

associated lymphoid tissues (BALT), and organized lymphoid tissue in the genitourinary tract [5].

In the MALT complex mucosal vaccine antigens are captured by antigen-presenting cells, APCs (Dendritic cells, B cells, Macrophages). They are then internally processed and presented on the cell surface by the MHC classes I and II, which are recognized by naïve $CD8^+$ and $CD4^+$ T cells respectively. The migration of lymphocytes from inductive sites to target effector sites is largely determined by site-specific integrins or 'homing receptors' on the lymphocyte surface and complementary mucosal tissue-specific adhesion molecules or 'addressins' on vascular endothelial cells at the effector sites. Cell migration is additionally controlled by chemokines produced in the local microenvironment which attract circulating mucosa-derived lymphocytes to attach to the endothelial addressins for subsequent exit across the endothelium into the mucosal tissue [5, 39, 40].

For example, in the GALT oral antigens induce effector or memory cells (both B and T cells) expressing α4β7 integrin, which attaches to mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), and CC chemokine receptor 9 (CCR9), which is the receptor for the chemokine ligand 25 (CCL25). MAdCAM-1 is expressed in microvascular endothelial cells of the gastrointestinal mucosa (but not in respiratory or genital mucosal tissues) where it supports leukocyte adhesion, and CCL25 is selectively present in the small intestine, where it plays an important role in the intestinal homing of IgA-committed B cells and antibody-secreting cells [41, 42].

An important characteristic of the mucosal adaptive immune response is the local production and secretion of dimeric IgA, which through its passage through the mucosal epithelium associates with "secretory component" protein to appear as secretory IgA (SIgA) in mucosal secretions [5]. SIgA has multiple roles in mucosal defence. It promotes the entrapment of antigens or micro-organisms in the mucus, thus inhibiting their direct contact with the epithelium and preventing their breaching the mucosal barrier. SIgA also inhibits the colonization and multiplication of mucosal pathogens, can neutralize viruses and toxins and may even neutralize pathogens that have breached the epithelium [43-46].

Cell-mediated immune responses are mediated by intraepithelial and lamina propria $CD4^+$ helper and Cytotoxic T lymphocytes (CTLs) which have a role

in clearance or containment of mucosal pathogens that enter the host via mucosal surfaces. Th1 and Th17 cells are found in the lamina propria throughout the gastrointestinal tract [47], and studies have identified an important role for Th17 cells secreting IL-17A in promoting sIgA production and secretion [48]. Long-term memory T and B cells can be rapidly recalled to mucosal surfaces upon reappearance of the antigen, where the B cells can rapidly evolve into IgA-secreting plasma cells [49].

TYPES OF VACCINES

Vaccines can broadly be grouped based on their composition and on their mode of administration. Vaccine composition may consist of either live attenuated or inactivated/killed bacteria or viruses or of specific microbial components (subunit vaccines). With regard to mode of administration, vaccines may be classified as parenteral vaccines given by intramuscular, subcutaneous or intradermal injection or as mucosal vaccines given by oral, nasal or more rarely sublingual, pulmonary, rectal or vaginal administration. A further classification may be whether or not the vaccines are presented together with a specific adjuvant [50, 51].

Live attenuated vaccines

Live vaccines are made using 'wild-type' viruses or bacteria that have been attenuated to become less virulent than their wild-type parental strains. Because live attenuated vaccines retain many of the intrinsic characteristics of the wild-type strains, they are able to colonize and multiply without causing the disease, thereby providing sufficient amounts of antigen and an appropriate selection of PAMPs to promote a protective immune response [52, 53].

However, removal of virulence genes, and the construction of vaccine strains which are safe and genetically stable is often a multi-step process. The balance between the extent of attenuation and retained immunogenicity is often a challenge in vaccine development. The serial cultivation technique as means to achieve attenuation of a pathogen *in vitro* or in non-habitual hosts was originated with Calmette and Guérin, who passaged bovine tuberculosis bacteria 230 times in artificial media to obtain an attenuated strain to protect against human tuberculosis. Whereas the attenuation of the oral vaccine against *S. Typhi* was achieved through selected gene deletion, resulting in a good safety

profile and retained immunogenicity [5, 53]. Examples of genetic engineering techniques for the production of live attenuated vaccine strains are the cholera strains CVD 103-HgR and Peru-15 both of which have been extensively modified in order to reduce pathogenicity and reactogenicity [54].

Killed or subunit vaccines

Non-live vaccines can contain whole pathogens or only components of them such as proteins or polysaccharides (subunit vaccines). Since they do not contain any living or infectious particles, these vaccines generally have a good safety profile even in immunocompromised individuals [55].

Subunit vaccines can be toxoid vaccines containing toxins detoxified by heat, chemicals (e.g. formaldehyde) or other means. Once inactivated and no longer pathogenic, they can still retain the ability to induce toxin-neutralizing antibodies. Classical examples are diphtheria and tetanus toxoid vaccines derived from *Corynebacterium diphtheriae* and *Clostridium tetani*, administered via intramuscular or subcutaneous injection [56-58].

Polysaccharide vaccines consist of purified polysaccharides from bacterial strains where these molecules are present on the bacterial surface creating a capsule which allows the bacteria to survive in the body. First-generation vaccines against *Streptococcus pneumoniae, Haemophilus influenzae* type b and *N. meningitidis* were based on capsular polysaccharides purified from whole pathogens, such as the 23-valent pneumococcal polysaccharide vaccine that was licensed in 1983 (Pneumovax 23, MSD; PNEUMO 23, Sanofi Pasteur). However, polysaccharide vaccines are poorly immunogenic, especially in young children, provide only short term protection, and can lead to reduced immune responses after repeated vaccinations (hypo-responsiveness) [59, 60]. Polysaccharide conjugate vaccines consisting of purified polysaccharides coupled to a protein. This transforms the T-cell-independent response induced by polysaccharides into a T-cell-dependent response that induces high-affinity antibodies and immune memory [61] and, given parenterally, have enhanced immunogenicity and are also effective in young children. Although promising, currently no licensed conjugate vaccines for mucosal administration exist.

Killed bacterial or viral vaccines are based on whole pathogens inactivated by heat, irradiation, or chemicals such as formalin or phenol. Inactivation destroys the pathogen's ability to replicate and cause disease but largely maintains its immunogenicity retaining protective antigens and innate immunity-stimulating PAMPs. Inactivation approaches were first used to create vaccines against pathogens such as typhoid fever, plague and cholera [62].

MUCOSAL VACCINES

As already mentioned in the introductory overview, most infections gain entry through mucosal surfaces. It can therefore be argued that mucosal immune defence is best stimulated by topical mucosal vaccines and there are clearly potential logistic advantages of the mucosal administration route.

However, although a mucosal route of vaccination is in general the best way to induce protection against mucosal infections, there are several situations when a parenteral vaccine may also work. Clearly this is the case with those mucosal infections where the pathogen causes disease only after having left the mucosa and entered into the blood or other organs. This situation applies to e.g. typhoid fever, polio and influenza, and for these infections there exist effective parenteral vaccines in addition to the mucosal ones. Likewise, the severe invasive infections caused by encapsulated bacteria such as pneumococci, meningococci and *Haemophilus influenzae,* start as mucosal infections but cause life-threatening disease after entering the blood-stream. Not surprisingly therefore there exist highly effective injectable polysaccharide or conjugate vaccines against these infections which give rise to high levels of opsonophagocytic antibodies (mainly IgG).

Another situation is when the mucosal infection occurs at a mucosal surface which, especially when it is inflamed during infection, is permeable to serum antibodies. The mucosal surfaces of the lower respiratory tract and of the vagina are examples of such "leaky" mucosal tissues; hence the injectable pneumococcal vaccines can protect not only against septic pneumococcal disease but also against lung pneumonia, and the injectable human papilloma virus (HPV) vaccines against genital HPV infection. In contrast, the healthy uninflamed mucosa of the gastrointestinal tract is much tighter and does not allow serum antibodies to reach the intestinal surface; in consequence, only

oral-mucosal immunization is effective in stimulating protective immunity against non-invasive, non-inflammatory gastrointestinal infections such as cholera and ETEC diarrhea in previously unprimed individuals [63].

However, even for the latter type of infections, parenteral vaccination may elicit a mucosal immune response in individuals that have been previously repeatedly exposed mucosally to the vaccine or pathogen. This was shown initially for cholera and polio [64, 65] and later also for other infections and vaccines. This explains why the now abandoned injectable cholera vaccines gave rise to measurable immunity in older individuals in cholera-endemic areas but not in young children, and why injectable polio vaccine (IPV) can elicit not only a protective serum IgG antibody response but also polio virus blocking sIgA immunity in the intestine [66].

REMAINING CHALLENGES

Vaccine efficacy faces several challenges related to impaired immune responsiveness in the whole or part of the target population. Here we discuss some of the principal causes, such as age, malnutrition, and immunosuppressive infections (e.g. HIV), and the possible reasons behind vaccine failure in these subjects.

In developing countries, primary infant immunizations are typically administered at 6, 10 and 14 weeks of age to stimulate protection against *B. pertussis* and other infections that pose risks in early life. However, young infants are difficult to immunize because of immunological immaturity and blocking effects of maternal antibodies. Vaccines also tend to be less immunogenic in elderly people compared to younger adults. This is the result of a progressive age decline of innate and adaptive immune responses which increases the frequency and severity of infections and reduces the protective effects of vaccinations. It is thus important to design vaccination strategies specifically tailored to these vulnerable populations, both in terms of vaccine formulation and vaccination protocols. Strategies to enhance vaccine-induced protection in the very young and in the elderly include the use of higher vaccine doses and/or specific adjuvants [67, 68].

Malnutrition increases susceptibility to infections. In malnourished individuals, dysfunction of innate and adaptive immunity has been described.

However, it appears that malnourished children can generally mount protective responses to both killed whole-cell and protein and polysaccharide subunit vaccines; although antibody titers and affinity are sometimes lower than in well-nourished children. T cells appear to be particularly affected by malnutrition and responses to live T cell dependent vaccines such as BCG may be suboptimal. Animal data also suggest that memory maintenance is impaired in malnutrition, which has important implications for long-term protection from vaccination [69, 70].

In patients with immune deficiency, the safety and efficiency of vaccines vary with the type and severity of immunosuppression. Although the protective antibody levels achieved in healthy individuals cannot be attained in patients with immune deficiency, there is no drawback in administering inactivated vaccines in accordance with the vaccination program. On the other hand, live viral and bacterial vaccines should not be administered to patients with serious immunodeficiencies since they could cause to systemic infection [55].

While these limitations apply to both parenteral and mucosal vaccines, there are also additional challenges specific for mucosal vaccines, especially orally administered live attenuated vaccines. These will be discussed in the next section.

SPECIFIC CHALLENGES FOR MUCOSAL VACCINES

One of the challenges for mucosal vaccine development is the need to overcome the mechanism of tolerance or unresponsiveness. Mucosal tolerance is a natural regulatory mechanism which prevents harmful inflammatory responses to environmental antigens. A particular problem with mucosal antigen exposure is thus the risk of inducing mucosal tolerance rather than protective immunity [5, 53]. Depending upon the dose of antigen administered, deletion or anergy of antigen-specific T cells and/or expansion of regulatory cells producing immunosuppressive cytokines (IL-4, IL-10), may result in decreased T-cell responsiveness. The dominant mechanism in mucosal tolerance is the induction of T regulatory cells (Tregs) following antigen exposure, which through secretion of TGFβ and/or IL-10 can inhibit the activation and function of helper and effector T cells [71].

Another challenge is the reduced immunogenicity of mucosal vaccines when used in developing countries, especially orally administered live attenuated vaccines such as polio, rotavirus and live cholera vaccines. This phenomenon which has been referred to as the "tropical barrier for mucosal vaccines" is attributed to chronic environmental enteropathy (also called tropical enteropathy) characterized by malabsorption associated with chronic intestinal inflammation possibly caused by poor sanitation and a harmful intestinal flora [72]. In addition, nutrition-related factors, including both protein-calorie and micronutrient malnutrition, may negatively impact on mucosal vaccine immunogenicity. This is also true of interference from maternal antibodies during breastfeeding, intestinal parasitic infections, intestinal mucosal damage and possibly maternal malnutrition during pregnancy. Lastly, host genetic factors may also contribute to the observed differences in responsiveness to mucosal (and other) vaccines in different populations [63, 73].

Vaccines designed for oral administration will need to be adjusted to these potential problems in order to maximize benefits for all children. In order to achieve this, oral vaccines when given to children in developing countries may require specific measures to such as higher doses of vaccine. Other strategies that have been tried with promising results are additional booster doses; nutritional supplements; withdrawal of breast milk before vaccine administration; and de-worming medications [66, 74].

A further challenge for mucosal vaccines is the often limited uptake of vaccine antigens across the mucosal barrier into the underlying MALT inductive sites. The vaccine antigens may be diluted in mucosal secretions, captured in the mucus layer, attacked by proteases and nucleases, and excluded by epithelial barriers [46]. As a result, much larger doses of vaccine are often required compared to parenteral vaccines. This increases costs even though mucosal administration as such is safer, simpler and cost-saving. There is a need for improved mucosal delivery systems to overcome these challenges and as discussed below co-administration with an adjuvant may often also be needed to overcome these limitations.

ADJUVANTS – A TOOL TO OVERCOME MANY VACCINE BARRIERS

DEFINITION AND MODE OF ACTION

As its name suggests, an adjuvant (derived from the latin *adjuvare* meaning "to help") enhances the immune response to co-administered vaccine antigens. Addition of an adjuvant to a vaccine can lower the amount of antigen required and/or reduce the number of immunizations. Adjuvants have also been found to improve the efficacy of vaccines in vulnerable populations [75].

The adjuvant effect was first observed in horses that developed abscesses at the injection site when immunized with diphtheria toxoid. It was subsequently found that an abscess generated by the injection of unrelated substances along with the diphtheria toxoid increased the immune response against the toxoid [76]. Many adjuvants are strikingly potent, but also very harmful to the host. Therefore, the potency of an adjuvant often conflicts with host safety and tolerability.

The mechanisms of action of many adjuvants are still not fully understood but some of the possible modes of action are summarized in figure 1.

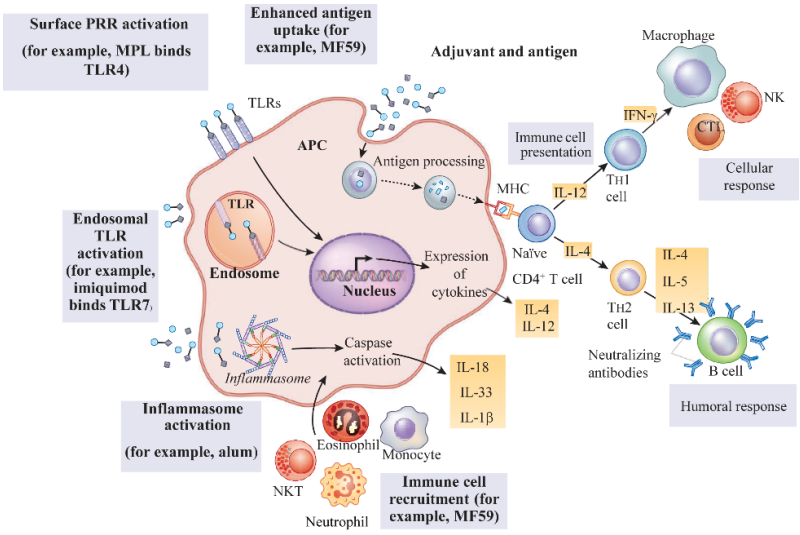

Figure 1. Putative mechanism of action of adjuvants. Figure adapted from PMID:24309663 [77].

Adjuvants can broadly be classified into two categories: 1) Immunostimulatory molecules derived from natural immune-stimulants (such as bacterial enterotoxins), Toll-like receptor (TLR) ligands (such as CpG oligonucleotide and lipopolysaccharide), and cytokines and 2) Delivery and/or depot-inducing vehicles with other forms of immunostimulatory activity. This category includes the most widely used vaccine adjuvant alum (aluminium hydroxide or sulphate) as well as saponin-based systems such as QS21; emulsions such as MF59 (an oil-in-water made of squalene); or microparticles such as virus like particles (VLPs). Several adjuvants, such as AS04 approved for use in HPV and Hepatitis B vaccines, may possess both immune-stimulating and antigen delivery properties [77].

Injected depot-forming adjuvants have been reported to prolong antigen delivery to APCs, induce inflammation and increase cellular trafficking and infiltration to the injection site. Furthermore, both injected and mucosal adjuvants have been found to promote the activation state of APCs by upregulating MHC expression and/or costimulatory signals, inducing cytokine release and enhancing antigen processing. Improved antigen presentation affects the speed, magnitude and duration of the immune response. Adjuvants have also been found to modulate antibody affinity and isotype as well as the magnitude of the antibody response and can promote induction of cell-mediated immunity and lymphocyte proliferation [77, 78].

Many adjuvants can act as ligands for PRRs activating an innate immune response. Receptor signaling can activate transcription factors that induce the production of cytokines and chemokines that help direct a particular immune response.
In terms of adjuvant action, the family of transcription factors collectively referred to as nuclear factor-kappa B (NF-κB) is thought to play a central role. NF-κB is formed through the homo- or hetero-dimerization of members of the Rel family of DNA binding proteins. They are activated by a variety of stimuli and in turn control expression of diverse genes involved in the immune response. NF-κB signal transduction mechanisms can be classified into the classical or the alternative (non-classical) pathways summarized in figure 2.

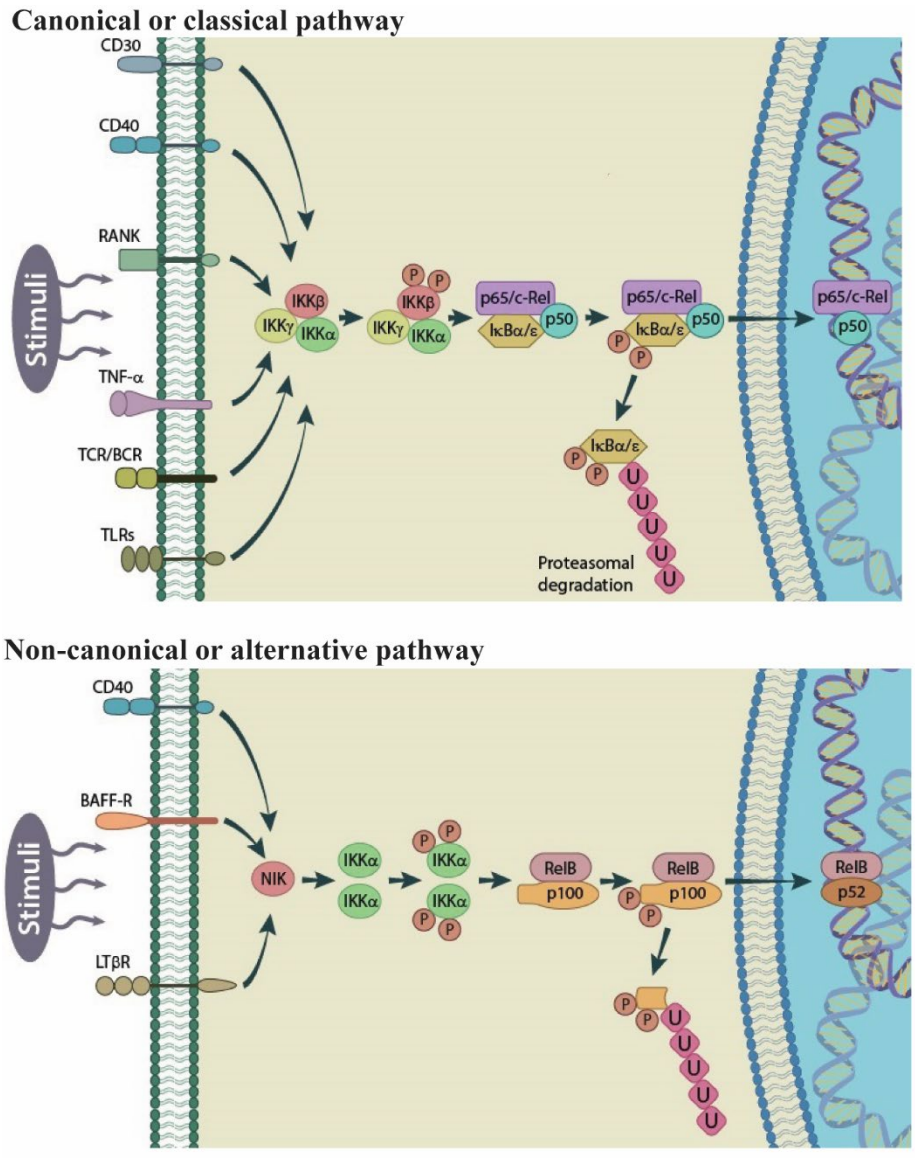

Figure 2. Schematic representation of the NF-κB activation pathway. Figure adapted from : https://www.mechanobio.info/what-is-mechanosignaling/signaling-pathways/what-is-the-nf-%CE%BAb-pathway/.

The classical NF-κB pathway (figure 2) is activated in response to pro-inflammatory stimuli, such as LPS, TNF, or CD40L [79], leading to activation of IKK (Inhibitor of Kappa B Kinase) complex, NF-κB heterodimer p50-

RelA/c-Rel release and nuclear translocation. Once in the nucleus the NF-κB complex binds DNA and increases (or reduces) transcription of NF-κB responsive elements. The alternative pathway, on the other hand (figure 2), is activated by members of the TNF-receptor superfamily, such as the lymphotoxin receptor, B-cell activating factor, and CD40, and is dependent on the induction of NIK (NF-κB-Inducing Kinase) signaling, leading to release and nuclear translocation of mainly p52-RelB dimers [80].

NF-κB signaling occurs largely at the level of APCs, usually through the interaction between PAMPs and membrane-bound or cytosolic PRRs and leads to enhanced expression of cytokines, chemokines and adhesion molecules important for APC activation and induction of an adaptive immune response. It is the NF-κB classical pathway that is most frequently activated through TLR signaling [81, 82]. Examples are TLR9 activation by the agonist CpG (a small oligodeoxynucleotide motif) which induces strong Th1 responses [83] and TLR4 activation by the agonist monophosphoryl lipid A (MPL) derived from LPS of Gram-negative bacteria, such as *Salmonella minnesota* [84]. Both CpG and MPL have been used as adjuvants in different formulations.

Another effect implicated in the action of some adjuvants is activation the inflammasome. This leads to the production of active proinflammatory cytokines, due to caspase activation which is responsible for proteolytic cleavage of inactive pro IL-1β and IL-18 into their active forms [85, 86].

MUCOSAL ADJUVANTS

The route of vaccination is important for a successful result, but of equal importance is the use of an appropriate formulation, especially when using non-living vaccines for mucosal immunization. The inclusion of an effective adjuvant is seen as crucial for effective mucosal immunization with subunit vaccines owing to tolerance being the natural "default" response induced to a soluble antigen at mucosal sites. This can be overcome by including an adjuvant in the vaccine formulation that provides signals that activate innate responses in mucosal epithelial and immune cells.

No mucosal adjuvants are as yet approved for routine clinical use. The experimentally most potent adjuvants for mucosal immunization are the bacterial enterotoxins CT and LT and their detoxified derivatives, TLRs

agonists [flagellin, poly(I:C), CpG ODNs], and a few other substances such as α-galactosylceramide and chitosan.

CHOLERA TOXIN AS MODEL FOR MUCOSAL ADJUVANTS

CT and LT enterotoxins are closely related both structurally and functionally and are to date, the most effective mucosal adjuvants in experimental animal studies. The 84 kDa holotoxins consist of a single 28 kDa enzymatically active A subunit non-covalently attached via its carboxy-end (A2) to a ring of five identical 11. 6 kDa cell-binding B subunits. The B subunit pentamer mediates binding to host cells via the mucosal cell-surface receptor galactosyl - N – acetylgalactosaminyl - (N – acetyl neuraminyl) –galactosyl glucosyl ceramide (GM1) [87] and other sugar based receptors [88, 89] and is not toxic.

For the A-subunit to exert its toxicity it needs to undergo proteolytic cleavage or 'nicking' between its A1 and A2 domains. In CT this is achieved by *V. cholerae* protease while nicking of LT is done by intestinal trypsin [90]. Following receptor-binding, the toxin is endocytosed and taken to the endoplasmatic reticulum (ER) by retrograde vesicular transport through the Golgi apparatus.

Once in the ER, the CTA dissociates from CTB and the disulfide bond, which links the CTA1 and CTA2, is reduced allowing the separation of the subunits. The translocation of CTA1 into the cytosol engages a natural recycling cellular process, so called ERAD (ER-associated degradation) or degradasome pathway which takes misfolded proteins from the ER to the cytosol where they will be degraded. CTA1 evades this fate by escaping ubiquitination and when in the cytosol it refolds and becomes fully active by binding ADP-ribosylating factor 6 (ARF6). The active CTA1 transfers the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD) to Gsα while GTP is bound. In this form it cannot hydrolyze GTP and as a consequence Gsα targets such as adenylate cyclase (AC), are irreversibly activated [91, 92].This results in dramatically increased cAMP levels. One of the consequences of this is the phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, causing a dramatic efflux of ions and water from crypt cells leading to the characteristic watery diarrhea [93]. It is however, not only the toxicity that is dependent upon the enzymatic activity of CTA1. The

adjuvant action is also strictly dependent on its ADP ribosylating properties [94].

Previous work by numerous groups has shown that CT promotes both cellular and humoral immune responses mainly via its action on APCs [95, 96]. CT upregulates the expression of MHC/HLA-DR molecules, CD80/B7.1 and CD86/B7.2 co-stimulatory molecules, as well as cytokines, on both murine and human DCs and other APCs [97, 98]. In mice, Mattson *at al*. clearly showed that DCs are the prime target cells for the adjuvant effect of CT, and that Gsα expression in these cells is required for the adjuvant activity [99]. Other studies have shown that CT via the APCs, can induce priming of Th1, Th2, Th17 and follicular helper T cells (Tfh) [98, 100-105]. In human cells it has also been widely reported that CT acts primarily on APCs and, as in murine APCs, it activates intracellular cAMP-protein kinase A (cAMP-PKA) and inflammasome-dependent pathways associated with expression, maturation, and release of IL-1β . This in turn enhances both humoral and effector T cell responses [99, 106, 107].

IL-1β is an important pro-inflammatory cytokine known to be induced via NF-κB signaling by several well-established adjuvants including lipopolysaccharide (LPS), aluminium hydroxide, and saponins [108-110]. Our previous work on human APCs has demonstrated that the CT-induced IL-1β primarily led to development of enhanced numbers of Th17 cells [107]. Consistent with this, Datta *et al*. [104] reported that CT-induced enhancement of mucosal IgA antibody responses in mice *in vivo* is dependent on IL-1 driven Th17 responses, and Hirota *et al*. [105] found a requirement for Th17 cells in the induction of T cell–dependent IgA responses in Peyer's patches. Mucosal immunization of CT with irradiated anthrax spores or ovalbumin in combination was shown to induce vaccine-specific Th17 cells [104].

In animal models, CT also acts as an effective mucosal adjuvant in vaccine-induced protection against a variety of pathogens. Examples include, tetanus toxoid [111] and Helicobacter pylori [112].

NEXT GENERATION CT- DERIVED MUCOSAL ADJUVANTS

CT and LT are the most potent known mucosal adjuvants, and have a number of properties that would make them excellent adjuvants. They are remarkably

stable to proteases, bile salts and other compounds in the intestine. They bind with high affinity to receptors present on most mammalian cells including the M cells covering the Peyer´s patches, as well as all antigen-presenting cells (APC). This facilitates the uptake and presentation of the toxins to the gut mucosal immune system. Finally, CT has strong inherent adjuvant and immune-modulating activities that depend both on its cell binding capability and its enzymatic ADP-ribosylating function [90]. However, because of their high toxicity they cannot be used in humans. Oral administration of CT caused profuse diarrhoea in human volunteers [113]. Administration of 2μg of LT with an intranasal influenza vaccine was found to cause Bell's palsy, which resulted in the rapid withdrawal of this adjuvant from the market [114].

To overcome the toxicity issues, several modified molecules derived from the native CT and LT have been developed and tested for their ability to enhance immune responses against co-administered antigens. LTK63, a mutant of LTA1 with a single mutation in the active site was reported to be safe in preclinical toxicological studies [115]. Nasal delivery of LTK63 along with influenza vaccine to human volunteers enhanced both Th1 and Th2 responses, however, as with native LT, Bell's palsy was observed in a few cases after vaccination and LTK63 is no longer considered to be safe for human use [116, 117]. Another genetically detoxified CT-based molecule with adjuvant activity is CTA1-DD derived from the fusion of CTA1 gene (responsible for enzymatic activity), with the Ig-binding dimer of the D-fragment from *Staphylococcus aureus* protein A. CTA1-DD binds to the Fc fragments of most immunoglobulins and forms an immune complex. Although not yet tested in a clinical setting, animal studies have shown that CTA1-DD toxicity is 100–1000 times less compared to whole wild-type CT [45, 99, 118-120].

Particularly promising adjuvants are based on mutations affecting the 'nicking' region of the A subunits of LT or CT, with the double mutant LT (LTR192G/ L211A, known as dmLT) being the most advanced with regard to clinical testing in humans. Convincing preclinical data have been generated for both these mutants, suggesting that toxicity and adjuvanticity can be effectively separated. Indeed, they were found to lack detectable enterotoxicity in mice, and exhibit much reduced cAMP-inducing activity but have retained significant adjuvant functions [121]. Subsequent clinical trials have demonstrated that dmLT is safe and non- toxic in humans [122, 123], and could

be a good adjuvant for mucosal vaccinations. In paper II, we introduce an additional non-toxic mucosal CT-derived adjuvant, which contains in its backbone sequences of amino-acids derived from the A subunit of LT.

OTHERS

Other adjuvants that have the potential to work mucosally include a variety of small molecule, non-TLR immunostimulants. Many of these molecules activate innate immune sensors on specific cell types. α-Galactosylceramide, a CD1d ligand and NK T cell activator, induced IgA in mice when used in an intranasal influenza vaccine [124, 125], a HIV vaccine given orally or intransally [126] but seems to suppress Th17 responses [127].

An additional vaccine formulation designed to enhance the immune response is the use of delivery systems consisting of microparticles, liposomes and other particulates. These are specifically designed to improve transport and release of the antigen payload. Particulate carriers can improve vaccine delivery by protecting antigens from gastric degradation, targeting the delivery to specific regions of the mucosa, inducing efficient uptake and Ag presentation by DCs, and/or controlling antigen release. The use of polymers for carrier preparation allows flexibility in the size, charge and surface properties of the vaccine formulation. An example of vaccine carriers are the biocompatible polymers. Poly (ethylene-glycol), poly(lactic-co-glycolicacid), chitin, chitosan, and their derivatives have been explored for delivery via the oral, intranasal and pulmonary routes. ETEC and Oral cholera vaccines have been incapsulated together in SmPillR-based oral delivery system where they were combined α-galactoceramide [128, 129]. Oil-based and lipid-based formulations have also been developed for mucosal and transcutaneous immunization. [50, 130]. Virus-like particles and viral vectors, such as adenovirus, have also demonstrated effectiveness in vaccine formulations [50].

2 AIMS

My thesis project can be divided in two parts. The first focuses on vaccine development, aiming at the construction and characterization of bacterial strains that can be used for antibiotic free production of vaccine antigens on the one hand and on the other, the construction from CT of a detoxified "safe" adjuvant for mucosal vaccines including oral cholera vaccine. The second part then aims at a further understanding of the molecular adjuvant mechanisms of CT and the non-toxic CT-derived molecule (mmCT).

Specific aims included the following:

1. To construct a non-antibiotic selection system for expression of recombinant vaccine antigens in *E. coli* or *V. cholerae* based on expression plasmids that are maintained by complementation an essential bacterial gene, and to validate the usefulness of the system for production of model vaccine antigens.

2. To construct, characterize and pre-clinically evaluate a novel detoxified mutant derivative of CT (mmCT) that can be used as an adjuvant for mucosal vaccines including oral cholera vaccine and that can be efficiently produced in genetically manipulated *V. cholerae*.

3. To investigate the molecular mechanisms of adjuvant action of the mmCT compared to CT using a range of different methods to focus on their effects on antigen-presenting cells.

3 METHODS

This section gives an overview of some of the methods needed to execute the current work. A more detailed description can be found in the published papers.

BACTERIAL STRAIN AND CULTURE CONDITIONS

V. cholerae and *E. coli* strains used in this thesis were all maintained on Luria Bertani (LB) agar plate supplemented when necessary with appropriate antibiotics, more information is found in respective papers. Strains were stored at −70°C in LB broth supplemented with glycerol (17% final concentration). Strains were grown at 37°C unless otherwise stipulated and liquid cultures were grown in rotary shakers (180 rpm).

GENETIC ENGINEERING OF BACTERIAL STRAINS

The generation of the bacterial strains carrying the *lgt* deletion, and the production strain for mmCT, were carried out using different genetic engineering techniques.

DNA MANIPULATION TECHNIQUES

E. COLI AND *V. CHOLERAE* EXPRESSION SYSTEMS

DNA fragments encoding the *lgt* gene and deletion derivatives were generated by PCR based techniques.

PCR primers were designed based on known sequences of the *E. coli* and *V. cholerae* genomes. All amplifications were performed using DNA templates obtained by boiling cells suspended in water. Whole genes were amplified using primers carrying convenient restriction sites at their ends in order to facilitate cloning. DNA fragments carrying the deletions that were eventually used for mutagenesis were generated by amplification of two fragments flanking the target gene which were then joined by primerless PCR.

All PCR products and the products produced by cloning were subjected to DNA sequencing; either of the plasmids directly or the PCR fragments in order to confirm that the sequences were correct. All sequencing was performed by a commercial contract service (Eurofins Genomics).

Cloning was done according to standard procedures in which DNA fragments digested with restriction enzymes, were ligated into appropriately digested plasmids. Bacteria were transformed using electroporation and selected either on the basis of antibiotic selection or temperature insensitivity.

Mutations were generated using a suicide vector system in which the target sequences were integrated into the chromosome together with the suicide vector by homologous recombination. The suicide plasmids were introduced into the recipient strains by conjugation. Selection for plasmid loss was done using the *sacB* gene from *Bacillus subtilis* present on the suicide vector. Clones losing the plasmid were chloramphenicol sensitive.

CONSTRUCTION OF mmCT

The strains were generated by re-insertion of the mutant *ctxA* genes into the classical biotype *V. cholerae* JS1569 strain that contains a deletion in the *ctxA* gene and has been used to express recombinant CTB [9].

A wild-type *ctxA* gene together with upstream DNA was amplified from the *V. cholerae* O1 El Tor strain Phil6973 [131] and cloned into a standard cloning vector. A BspEI/HindIII fragment carrying the two mutations in dmCT was synthesized and used to replace the 3_end of the native *ctxA* gene from the *V. cholerae* strain Phil6973. The mutant *ctxA* gene was assembled into a *ctxAB* operon using a previously cloned *ctxB* gene and this was inserted into a suicide vector [132]. The resulting plasmid (pSS-dmCT) was then mated into the *ctxA*-deleted recipient strain [9], for integration into the chromosome by homologous recombination. Following selection on sucrose plates, colonies which had lost the plasmid but had acquired *ctxA* expression were isolated, a representative strain being MS1405. The suicide plasmid pSS-mmCT was generated from pSS-dmCT by introducing additional changes using primerless PCR. The strain expressing mmCT (MS1559) was generated in the same way as the dmCT-expressing strain MS1405. In all cases cloned sequences were

confirmed by DNA sequencing before and after generating the final expressing strains.

PROTEIN PRODUCTION AND PURIFICATION

To characterize the strains in paper I, the mmCT in Paper II, and proceed with all the molecular studies in paper III and IV, the following step were performed to produce and purify the expressing proteins from the constructed strains.

Paper I focuses on studying whether the new constructed strains retain the same properties in terms of protein yield, and their folding abilities than the protein produced from wild type strains. In paper II describes the protein characterization performed to ensure the newly mmCT had a certain purity and binding properties similar to the native CT.

The CTB::p45 (fusion protein for vaccination against atherosclerosis) and GST (glutathione-S-transferase) proteins in paper I, were produced from the *E.coli* BL21 strain, in small and large scale depending by the experimental needs. Protein expression in *E.coli* BL21 is regulated by a Lac repressor, therefore the addition of 1mM IPTG was required to induce their expression. GST is a soluble protein, found in the cytoplasm, whereas CTB::p45 is insoluble and aggregates in inclusion bodies. Consequently CTB::p45 was recovered by centrifugation and extensively washed with 0.1% Triton X-114 and phosphate-buffered saline (PBS), before being dissolved in 6.5 M urea. Both proteins were reassembled by step-wise dialysis against sodium carbonate buffer (pH 9.0), and the assembly was checked by SDS-PAGE. The GST protein was purified by a HiTrap reduced glutathione affinity column, whereas the assembled CTB::p45 were purified by anion exchange chromatography using a Resource Q 6-ml anion exchange column. The protein was eluted with a linear gradient of 0 to 1 M NaCl in 50 mM carbonate buffer (pH 9).

In paper I, rCTB was produced in *V. cholerae* and the product was secreted into the growth medium from which it was purified. Medium for the optimal production of rCTB and the methods for purification are previously described [133]. In paper II, both dmCT and mmCT were produced on small scale using syncase medium and were incubated at 30°C for 20-24h. Larger batches of mmCT were produced in a 5 liter bioreactor, using the same medium and temperature conditions. Cultures were then harvested after 19h. Briefly, cells

were removed by centrifugation. The medium was retained and sterilized by filtration. Proteins were then precipitated by the addition of sodium hexametaphosphate and adjusting the pH to 4.5. The precipitates were collected by centrifugation and rCTB was re-dissolved in a minimal volume of 50 mM Tris-HCl pH 8. Non-dissolved material was removed by centrifugation followed by filtration and the resulting solution subjected to anion exchange chromatography using a Resource Q anion exchange column. Proteins were eluted using a sodium chloride gradient (0-0.5 M). When deemed useful, a final purification step was performed using gel filtration.

ELISAs

GM1 ELISA

To ensure that the newly rCTB/CT derived purified proteins were biologically active, a GM1 ELISA was performed. This ELISA assay was used to detect and quantify rCTB and its derivatives in Paper I, and to quantify the amounts of native CT, dmCT and mmCT produced from the different strains in Paper II. The GM1 ELISA assay was performed as previously described [134, 135] using a CTA-specific monoclonal antibody (CT17) and CTB-specific monoclonal antibody (LT39) [136]. The CT/CTB concentration in the samples was determined by comparison with the CT/CTB standard curve.

MEASUREMENT OF ANTIBODY LEVELS

Serum and mucosal antibody responses in paper II were determined by ELISA. High binding ELISA plates were coated with either OVA (1 µg/ml), *V. cholerae* O1 lipopolysaccharide (LPS) (5 µg/ml of a 50:50 mixture of LPS of Inaba and Ogawa serotypes in PBS [137]), or influenza virus HA (1 µg/ml). Samples and a standard of known activity included in each plate were titrated by threefold serial dilution. The different antibodies were detected using isotype-specific antibody conjugates carrying HRP (horseradish peroxidase).

SDS-PAGE AND WESTERN BLOT

In paper I, SDS-PAGE was performed to analyze the expression of recombinant proteins prior to and after purification. Samples were analyzed by SDS-PAGE under reducing conditions, boiled and in the case of CT and rCTB

derivatives non-boiled, with the latter being used to visualize the assembled proteins where needed (CTB, CTB::p45 and mmCT).

Western blotting was performed to visualize cholera toxin subunits using a ant CT monoclonal antibody [136] for the specific immuno-detection of CTA/CTA1 and CTB. These analyses were performed either directly or in the case of CTA derivatives, after incubation of the proteins with trypsin or spent culture medium from 30h cultivation of *V. cholerae* O1 classical strain JS1569 in syncase medium.

In paper III, this Western blot methodology has also been used to study the time points localization of NFκB transcription factor in the cellular compartments, cytosolic and nuclear, of THP1 monocytes cell lines. The detection was performed by using a polyclonal rabbit anti-p65 antibody for NFκB and the quantification was made possible by using densitometry analysis.

IN VITRO IMMUNOLOGICAL ASSAYS

cAMP

The main driver of toxicity in CT is the production of cyclic AMP (cAMP), which in intestinal cells cause diarrhea due to the mechanisms explained in the introduction. Therefore, cAMP is a good marker *in vitro* to study the levels of toxicity of CT-derived molecules.

cAMP was measured in mouse thymocytes following intoxication with serial dilution of purified toxin preparations using a commercially available kit (R&D Systems) based on an ELISA method. Sample preparation and determination of cAMP levels were done essentially in accordance with manufacturer's instructions. Single cell suspensions of murine thymocytes were treated with various concentrations of CT, mmCT, and CTB, or left untreated for 2½ hours. The cells were then lysed by freeze-thaw cycles, cell debris removed by centrifugation and cell lysates were used for analysis.

MONOCYTES - T CELLS CO-CULTURE

To study the mechanism of action, we used an established co-culture *in vitro* model [107], to measure the levels of IL17A produced by the T cells in response stimulation by CT or the mmCT. To verify whether the specific intracellular molecules were involved in adjuvanticity mechanisms, we used specific inhibitors to block protein activity, or silencing RNA (siRNA) to inhibit the transcription of specific target gene.

PBMCs were isolated from heparinised blood immediately after collection. For experiments using specific cell populations ($CD4^+$ T cells and $CD14^+$ monocytes), positive isolation and negative depletions were performed using immunomagnetic beads. $CD14^+$ monocytes were stimulated with 1μg/ml CT, mmCT, or left untreated for 16 h, and when used in co-culture experiments, subsequently washed with PBS three times, and mixed with autologous $CD4^+$ T cells with a polyclonal stimulus SEB (Staphylococcal enterotoxin B). Supernatants were collected after 72 h of co-culture, and subsequently stored at -70°C until analysis with ELISA for determining the levels of IL-17A.

For inhibition experiments (Paper III), monocytes were initially treated with 20μM H-89 (Dihydrochloride), or 20μM CAPE (Caffeic acid phenethyl ester) for 1 h, then subsequently stimulated with adjuvants for 16 h. For inhibition of specific gene expression small interfering RNAs (siRNAs) with specificity for the RELA and RELB genes (paper III) and THSB1 (Thrombospondin-1), ALOX-5 (Arachidonate 5-Lipoxygenase), ITGB1 (Integrin B1) (Paper IV) genes and the negative control ALL STAR siRNA was utilized. $CD14^+$ monocytes were treated with specific siRNA for 24 h at 37°C with 5% CO_2. Cells were then washed and subsequently incubated with 1μg/ml CT for 16 h. Following washing steps, cells were co-cultured with $CD4^+$ T cells, and analyzed for IL- 17A production as described above.

THP1 BLUE−NFκB CELLS

THP1 Blue−NFκB monocyte cell line purchased from InvivoGen, which carries a stable integrated NF-κB-inducible Secreted Embryonic Alkaline Phosphatase (SEAP) reporter construct, was used determine the presence of NF-κB induction in cells stimulated with adjuvants. THP1 Blue−NF-κB cells

were treated for 16 h with 1μg/ml of CT or mmCT or 1mM of the cAMP analog, dcAMP or left untreated in cell culture medium in 96-well plates. Inhibition of PKA was tested by adding 20μM H-89 1 h prior to the treatment with adjuvants. After incubation for 16 h, the cells were centrifuged at 350× g for 5min, and 20 μl of the cell supernatant was mixed with 180 μl pre-warmed SEAP detection reagent QUANTI-Blue (InvivoGen). After further incubation for 3 h at cell culture conditions, the levels of NF-κB-induced SEAP were measured.

FLOW CYTOMETRY

This technique allows us to discriminate cell populations from fresh PBMCs and evaluate the expression of selected cell–associated molecules; either surface targets or intracellular cytokines. Cells were incubated with antibody specific for surface molecular markers or cytokines conjugated with a fluorophore. Specifically, in paper III murine BMDCs were analyzed to evaluate the expression of CD80 and CD86 surface markers in CD11c cells following incubation with or without 1μg/ml of CT or mmCT for 16 h, with or without the prior addition of 20μM H-89. In paper IV, ITGB1 expression was measured in monocyte and dendritic cell populations after 16 h-treatment of fresh PBMCs with 1μg/ml of CT, mmCT, dmLT or media alone, with or without pre-treatment with PKA. Samples were analyzed with a LSRII Flow Cytometer (BD Biosciences), and data were then analyzed using FlowJo software (Tree Star).

PROTEOMICS STUDIES – CELL TREATMENTS

For proteomic analysis, PBMCs were isolated by Ficoll-Paque density gradient centrifugation from Buffy coats of 18 healthy human blood donors, followed by purification of $CD14^+$ monocytes by magnetic beads separation. Cells were treated with 1μg/ml CT for 2 h, 4 h, 6 h, and 16 h or incubated untreated for 2 h [non-stimulated (NS) controls] at 37°C with 5% CO_2. After 3 thorough washes with PBS, cells were snap-frozen in liquid nitrogen and stored at −70°C. Cells were transported in dry ice to Proteomics & Mass Spectrometry Facility, Donald Danforth Plant Science Center, Saint Louis, MO, USA for proteomic analysis. Cells were pooled so that each replicate was a pool of 6 individuals, yielding a total of 15 samples for analysis (triplicates of 4 CT

treatment time-points samples and 1 NS sample). Verification analysis was performed to ensure that cell counts did not change between the different time points, and between non-stimulated and CT-stimulated cells after 16 h. In addition, it was observed that trypan blue positive cells were consistently below 1%, indicating negligible cell death at all time points and treatments. This high number of cells was necessary to obtain the suitable number of cells for proteomic analysis and to account for individual variability among various samples.

IN VIVO IMMUNOLOGICAL ASSAY

MICE

In paper II and paper III, three animal models were used for different purposes. In paper II, mice were used to demonstrate immune response elicited by the newly-generated mmCT adjuvant administered together various candidate vaccines. In paper II, infant mice were used to evaluate the level of *in vivo* enterotoxicity of the tested adjuvant. In paper III, NF-κB knockout mice were used to determine the requirement of NF-κB signaling in the adjuvant action of CT.

ANTIBODY RESPONSE

Six to eight week old female C57Bl/6 mice were immunized either intranasally (i.n), or peroral/intragastric (p.o) (Figure 3) with Ovalbumin (OVA) administered alone, or mixed with mmCT or, for a comparison, CT as adjuvant. Mice were tested with OVA antigen mixed with adjuvant for studies of antibody response.

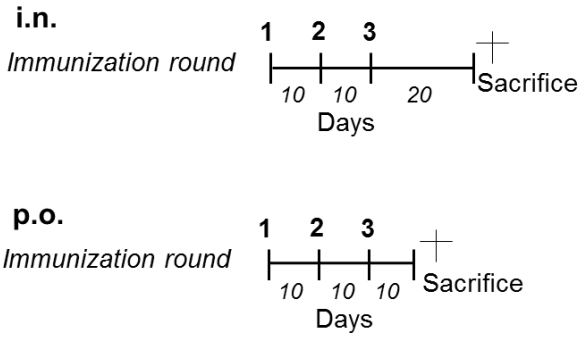

Figure 3. Intranasal and peroral immunization schedule to generate serum and mucosal antibody responses in mice. Mice were immunized in three rounds at ten days intervals. Mice were then sacrificed 20 days after the last i.n. or 10 days after the last p.o. immunization.

Inbred 6–8 week old female Balb/c mice were immunized p.o immunizations (Figure 4) with Dukoral administered alone or mixed with mmCT or, for comparison, CT as adjuvant. CB6F1/OlaHsd mice were tested sublingually (s.l) with HA antigen mixed with the adjuvant. The immunization schedules are shown in figure 4, and were performed to assess humoral antibody responses elicited by the adjuvant when administered with a mucosal vaccine.

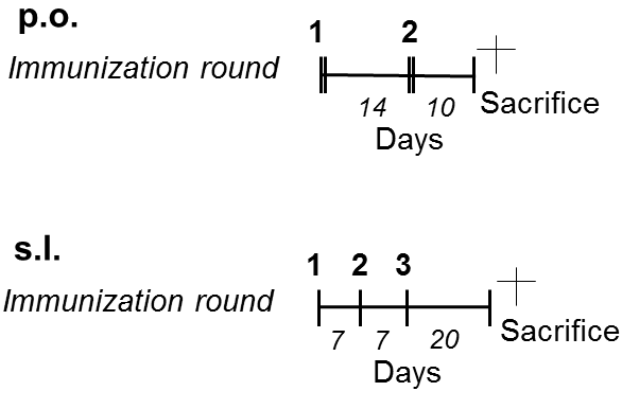

Figure 4. Peroral and sublingual immunization schedule to generate mucosal antibody response in .mice. In oral immunization, mice were immunized in two rounds at two-week intervals. Each round comprised two p.o. administrations on consecutive days. In sublingual immunization, mice were immunized three times at one week interval. Mice were then sacrificed 10 days after the last p.o. or 20 days after the last s.l. immunization.

T CELL RESPONSE STUDIES

To study the ability of mmCT to induce $CD4^+$ T cells, an expansional division of OT-II cells, derived for OT-II TCR transgenic mice, having almost 50% of their $CD4^+$ T cells, being specific for a defined OVA peptide epitope, was performed [138]. The experimental procedures of the adoptive transfer and immunization is described in the figure 5.

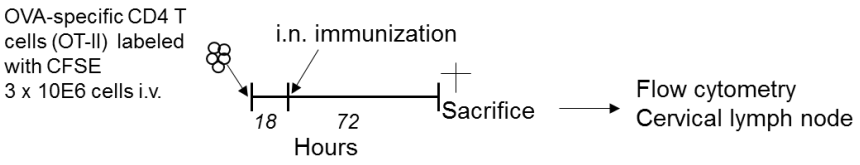

Figure 5. CD4 OT-II T cells labelled with CFSE (Invitrogen) 3–5 × 10^6 cells were injected i.v. into mice. Eighteen hours later the mice were immunized i.n. with OVA with or without mmCT or CT. Three days later, mice were sacrificed and then single-cell suspensions from cervical lymph nodes were analyzed by flow cytometry to determine the frequency of adoptively transferred cells that had entered division as described [139].

To test ability for the adjuvant to activate $CD8^+$ T cells, an *in vivo* cytotoxicity test was performed and shown in figure 6.

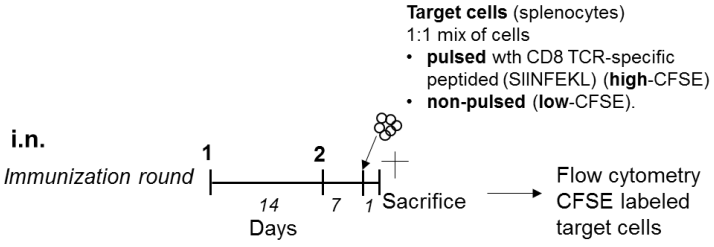

Figure 6. Mice were immunized i.n. twice at a two weeks interval with OVA ± mmCT or CT. One week following the last immunization, 8×10^6 donor C57BL/6 splenocytes were transferred i.v. to the immunized mice. Donor splenocytes comprised a 1:1 mix of cells pulsed with OT-I-specific peptide OVA257–264 (SINFEKL) and stained with high -CFSE dose, and non-pulsed cells low-CFSE dose. One day after adoptive transfer, splenocytes were analyzed for the presence of CFSE-labeled cells by flow cytometry. The percent-specific lysis was determined by loss of the peptide-pulsed CFSE high population compared with the unpulsed CFSE low population.

ENTEROTOXICITY – INFANT MICE

Three-day-old C57BL/6N mice (ranging from 2.3-2.7 grams in weight) were used to test *in vivo* enterotoxicity of graded doses of mmCT, CT and CTB as previously described [140].

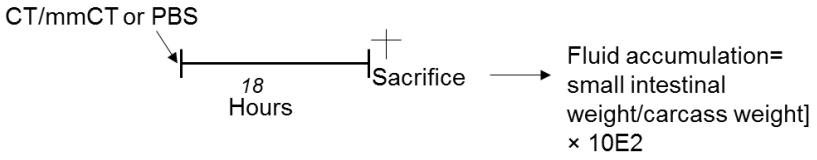

Figure 7. mmCT, CT and CTB were tested in three days old C57BL/6N mice (2.3-2.7grams) as described [140]. At 18 hours after inoculation the animals were weighed individually. After sacrifice their small intestines (pyloric valve to ileal-cecal junction) was removed in one piece and weighed. The small intestine-to-carcass weight (expressed as percentage) was determined as a measure of fluid accumulation.

4 RESULTS AND DISCUSSION

The results of all the included papers will be described and discussed in a rather broad context. Papers I and II concern vaccine development, including production strategies and the generation of a novel adjuvant. Papers III and IV examine the mechanism of adjuvanticity of the newly developed mmCT, whilst Paper II describes comparison studies between mmCT and CT. For a more detailed description of the results and their discussion, please refer to the papers.

PAPER I: DEVELOPMENT OF A NOVEL NON-ANTIBIOTIC, *LGT*-BASED SELECTION SYSTEM FOR STABLE MAINTENANCE OF EXPRESSION VECTORS IN *E. COLI* AND *V. CHOLERAE*

For reasons already discussed in this thesis, the use of antibiotics in the production of recombinant proteins is becoming increasingly discouraged. Therefore, using molecular biology techniques we were able to develop an antibiotic-free based expression selection system to be used in the making of protein subunit vaccines.

The *lgt* gene encoding a (pro) lipoprotein glyceryl transferase, an essential gene encoding for the biosynthesis of bacterial lipoproteins, was removed from the chromosome of the *E. coli* strain BL21 and the corresponding *lgt* gene from *V. cholerae* was used for complementation.

To generate the parental *lgt*-deleted strain the essential gene was provided *in trans* before it was deleted from the chromosome. The resulting recombinant *E. coli* strain produced was called MMS1742. The gene complementing the deleted *lgt* gene in MMS1742, is derived from *V. cholerae* and is therefore not homologous to the *E. coli* gene, even though it fulfils the same function. Thus, the only target for mutagenesis was located in the chromosome, and following deletion of the gene, there was little chance for reversion through recombination.

Importantly, the plasmid used for maintenance of the *lgt*-deleted strains is temperature sensitive. This means that it is straightforward procedure to replace it with an expression vector that is not temperature sensitive by selection of transformants at high temperature. Although this system is not full-proof since we often obtained backgrounds of colonies carrying maintenance plasmids that were no longer temperature sensitive, we were able to produce a number of different strains expressing recombinant proteins to high yields.

EXPRESSION OF RECOMBINANT PROTEINS IN Δ*LGT* STRAINS OF *E. COLI*

We constructed expression plasmids carrying the complementing *lgt* gene from *V. cholerae* and genes for model recombinant proteins. The proteins chosen were sj26GST; a soluble and biologically active glutathione-S-transferase derived from *Schistosoma japonicum* and CTB::p45, a fusion protein between the cholera toxin B subunit and a peptide derived from human low-density lipoprotein (LDL) produced as insoluble cytoplasmic inclusion bodies.

Proteins were expressed from the *tac* promoter under the control of the LacI repressor in small-scale cultures, and expression was induced by the addition of IPTG to the growth medium. The results shown in figure 8, demonstrate that both proteins expressed when cultures were induced.

Expression vector stability was tested using a plasmid encoding catechol 2, 3 dioxigenase, *dmpB*. *lgt*-deleted strains carrying the *dmpB* gene turned yellow due to the production of 2-hydroxysuccinyl semi-aldehyde when sprayed with a 10mg/ml solution of catechol. The final strain was passaged in liquid culture for over 40 generations and all the colonies picked expressed C23O (yellow colour) indicating full retention of the plasmid in the absence of antibiotic selection.

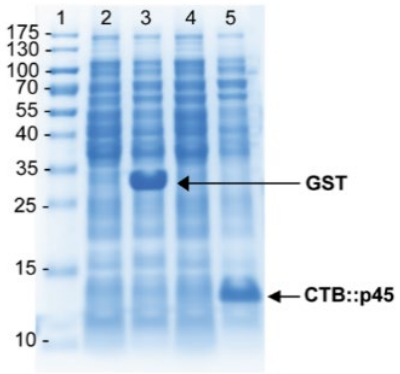

Figure 8. High-level expression of recombinant proteins in lgt-deleted strains. SDS-PAGE gel of protein lysates of the E. coli BL21-derived lgt-deleted strain MMS1742 carrying expression plasmids expressing different recombinant proteins. Lane 3 shows the expression of soluble recombinant sj26GST in strain MMS1808 (MMS1742/pMT-sj26GST/lgtVc), and lane 5 shows the expression of recombinant CTB::p45, which is insoluble and produced as inclusion bodies in strain MMS1762 (MMS1742/pMT-CTB::p45/lgtVc).

We also showed in comparative growth experiments that the *lgt*-deleted strains showed no significant difference in either growth or protein expression compared to strains maintaining plasmids using antibiotic selection markers (figure 9).

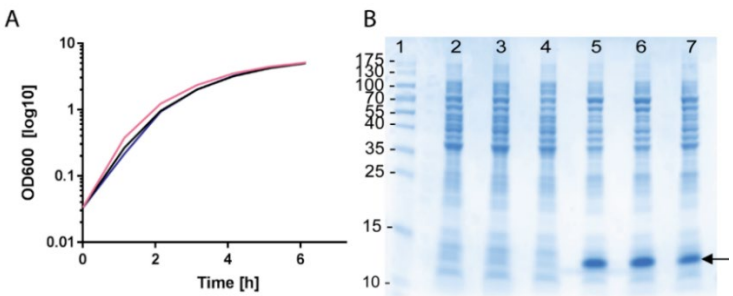

Figure 9. Comparable growth and protein expression levels in an E. coli Δlgt strain compared with the wild-type strain carrying a conventionally maintained plasmid. (A) Duplicate growth curves of an E. coli BL21 Δlgt Tn5 (kan) strain MMS1742 derivative carrying pMT-CTB::p45/lgtVc (pink and blue lines) and wild-type strain E. coli BL21 carrying the equivalent plasmid pML-CTB::p45 maintained by Amp resistance (black line). (B) SDS-PAGE showing expression comparisons of the same strains grown under inducing (lanes 5 to 7) and non-inducing (lanes 2 to 4) conditions. Lanes 2 and 5 and lanes 3 and 6 show protein from duplicate cultures of

the MMS1742 derivatives grown under inducing and non-inducing conditions, respectively, whereas lanes 4 and 7 show the parental BL21 strain carrying plasmid pML-CTB::p45 grown under inducing and non-inducing conditions, respectively.

Lastly, both the glutathione S-transferase (sj26GST), an active soluble protein accumulating in the cytoplasm [141] and the insoluble protein rCTB::p45 found in inclusion bodies, were produced using the *lgt*-based system, purified and their functional properties tested. The sj26GST was successfully purified by affinity chromatography based on reduced glutathione, hence indicating that the expressed protein has its expected function. The CTB::p45 was reassembled *in vitro* into a pentameric fusion protein as shown in figure 10. To ensure the CTB structure was restored, and binding to GM1 ganglioside in an enzyme-linked immunosorbent assay (ELISA) confirmed that the receptor binding properties of CTB were retained. We further showed that protein production could be scaled up and was not impaired when the strain was grown a large culture (3 L fermenter), suggesting that such strains could be used in industrial production of recombinant proteins. In this regard CTB::p45 was produced in 3 L fermenter and the protein was characterized as explained before.

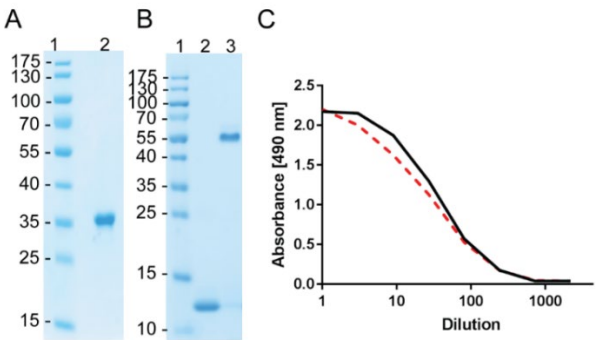

Figure 10. Purification and demonstration of the functional activity of recombinant proteins expressed as soluble cytoplasmic or inclusion body proteins in E. coli. (A) SDS-PAGE analysis following purification of sj26GST. Lane 2 shows the GST protein after binding to a GST Hi-trap column and elution with 10 mM reduced glutathione. (B and C) Purification of CTB::p45 from inclusion bodies (B) and determination of biological activity by a GM1 ELISA (C). (B) Coomassie-stained SDS-PAGE gel showing purified CTB::p45 expressed from E. coli Δlgt strain MMS1762 in denaturing conditions (lanes 2) and non-denaturing conditions (lanes3) (C) GM1 ELISA of CTB::p45 produced from MMS1762 (black line) and MMS1089 (broken red line) after

reassembly and purification. The presented data represent results from duplicate assays. The starting concentration of each protein was 0.5 µg/ml.

PARALLEL CONSTRUCTION OF *LGT*-DELETED V. *CHOLERAE* AND PRODUCTION OF RECOMBINANT CTB

As a further proof of concept, we generated an *lgt*-deleted strain of *V. cholerae*, by complementing *in trans* with the corresponding *lgt*-gene from *E. coli*. The production of CTB in *V. cholerae* is an example of constitutive expression from the *tac* promoter. The B subunit of Cholera Toxin (rCTB), was produced in large culture, and biological activity measured via GM1-ELISA. The results showed that the rCTB production yield and biological properties were the same as those obtained in the currently used system for industrial production of this protein (figure 11).

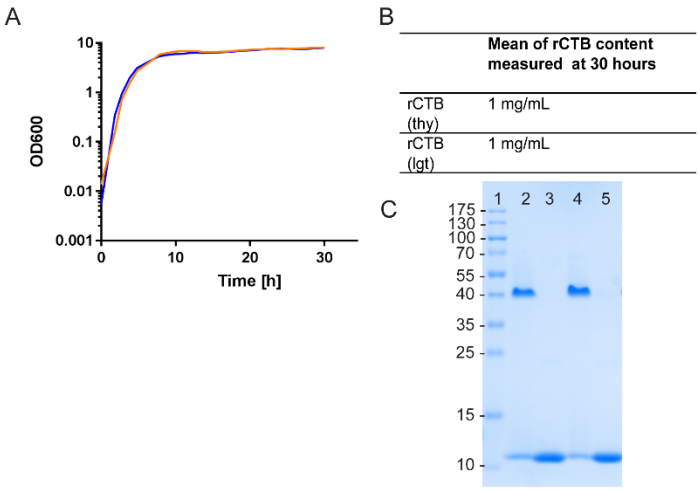

Figure 11. Comparable growth and expression of rCTB in different V. cholerae strains. (A) Growth curves of MS1012 expressing rCTB from a plasmid maintained by complementation of the thyA gene and MMS1692 expressing rCTB from a plasmid maintained by lgt complementation. (B) rCTB production in milligrams per milliliter, measured by a GM1 ELISA after 30 h. (C) Coomassie-stained SDS-PAGE gel showing rCTB in native (pentameric) and denatured forms from MMS1692 (lanes 2 and 3, respectively) and MMS1012 (lanes 4 and 5, respectively).

Overall, we conclude that the described *lgt* system appears highly promising for non-antibiotic dependent production of pharmaceutical proteins including vaccine protein antigens. Importantly, the growth of the *lgt*-deleted strains was not compromised in comparison with the parental strains, and the plasmids have proven to be extremely stable without the need for specialized media or supplementation with antibiotics. Likewise, the proteins expressed in *lgt*-deleted strains are functionally active, as shown by the retained activity-dependent purification of GST, the enzymatic activity of C23O in the plasmid stability test, and the pentamer assembly and GM1 receptor binding of both CTB::p45 and native CTB produced in the *lgt* system. Furthermore, the final yield of the recombinant protein was also fully comparable with that produced by a more conventional industrially used production plasmid. Since the *lgt* gene is essential in all Gram-negative bacteria, it should be possible to use this strategy for the production of recombinant proteins in other species.

PAPER II: mmCT CONSTRUCTION AND *IN VITRO* AND *IN VIVO* CHARACTERIZATION

There is a great need for mucosal adjuvants which are safe and can enhance the immunogenicity of mucosal vaccines, especially purified vaccine antigens that are often poorly immunogenic without adjuvanticity.

mmCT IS STABLE TO BOTH TRYPSIN AND BACTERIAL PROTEASES

In the present study we generated a non-toxic cholera toxin derivative called mmCT (multiple mutated Cholera Toxin) based on similar detoxifying mutations as in the adjuvant-active dmLT derivative from *E. coli* heat-labile toxin. Initially we made the same mutations as those present in dmLT, at amino acid positions 192 and 211(essentially to prevent nicking between the A1 and A2 portions of the CTA subunit). However, the resulting dmCT was still cleaved by *V. cholerae*-derived bacterial proteases despite being insensitive to trypsin. Mass spectrometry analysis revealed that in CTA at amino acid position 196, there was another proteolytic cleavage site.

Further, incubation of dmCT and dmLT in spent *V. cholerae* culture medium showed that dmCT was rapidly degraded whereas dmLT was stable. Thus, to generate a molecule that was resistant to *V. cholerae* protease cleavage and yet still assemble properly with CTB, we constructed a mutant CTA gene in which the region in CTA between the two cysteines (189-196 aa) was substituted with that from LTA. The resulting mmCT proved to be resistant to both trypsin and to *V. cholerae* bacterial proteases. Next, we were able to produce and purify the mmCT protein, and demonstrate its ability to bind to GM1 ganglioside in an ELISA assay. Proteins were produced in a 5-L fermenter and precipitated from the growth medium with sodium hexametaphosphate, dissolved, and subjected to SDS–PAGE and Western blot analysis using CTA-specific antibody (figure 12). mmCT was purified following two purification steps, an anion exchange chromatography and gel filtration.

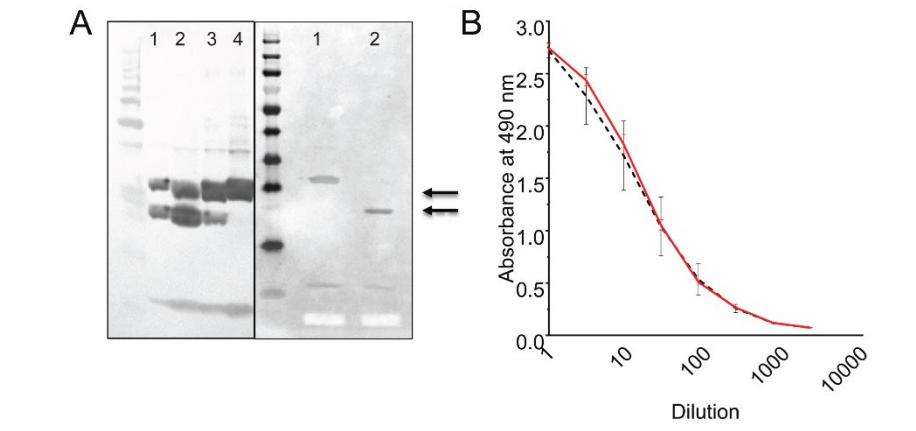

Figure 12. A) Western blot analysis using CTA-specific antibody CT17. Coomassie-stained SDS–PAGE and Western blot analysis (left) are shown for Lanes: (1) CT (List) standard, (2) CT from wild type strain 569B, (3) dmCT from strain MS1405, and (4) mmCT from strain MS1559. Western blot (right) of (1) the purified mmCT, compared with (2) purified CT (List) standard. In (A) the lower Western blot bands (dotted arrow) is the cleaved CTA1 molecule; the upper band (filled line arrow) is the non-cleaved CTA protein comprising both CTA1 and CTA2.B) GM1 ELISA of native CT (dotted line) and purified mmCT (solid line). The presented data represents duplicate assays. The starting concentration of each toxin was 0.5 μg/ mL.

mmCT LACKS ENTEROTOXIC ACTIVITY AND cAMP PRODUCTION ACTIVITY COMPARED TO CT

Since lack of enterotoxigenic (diarrheal) activity is essential for a candidate orally administered adjuvant for human use, we tested the toxic activity of mmCT, by measuring its ability to generate fluid accumulation in infant mice compared to CT, CTB and PBS. The results showed no toxic activity even when tested at a dose 100-fold higher than the dose of native CT causing significant intestinal fluid accumulation.

cAMP levels (reflecting ADP-ribosylating enzymatic activity associated with enterotoxicity) in mice thymocytes intoxicated with mmCT, showed a > 10,000-fold reduction in activity compared to native CT (figure 13).

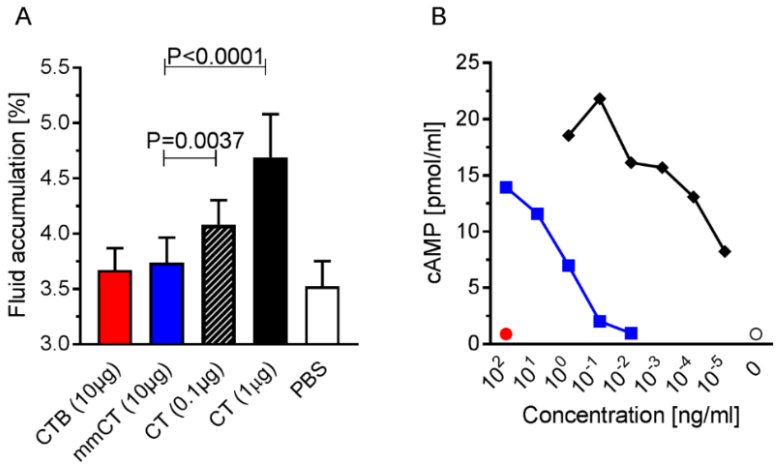

Figure 13. Toxicity testing of mmCT. (A) Fluid accumulation (FA) in infant mouse intestines in vivo (calculated using the formula FA = [small intestinal weight/carcass weight] × 10^2) after inoculation with mmCT as compared with CT or CTB. (B) cAMP production in mouse thymocytes in vitro after exposure to different concentrations of mmCT (Blue line), CT (Black line) or CTB (red dot).

STRONG MUCOSAL ADJUVANT ACTIVITY OF mmCT ON CELLULAR AND ANTIBODY RESPONSES TO A MODEL PROTEIN ANTIGEN

We examined the adjuvant activity of mmCT both for mucosal and systemic Ab responses as well as $CD4^+$ and $CD8^+$ T cell responses to a model antigen (OVA). A similar adjuvant effect could be demonstrated for all of these immune responses when mmCT was co-administered with OVA either nasally or perorally (figure 14).

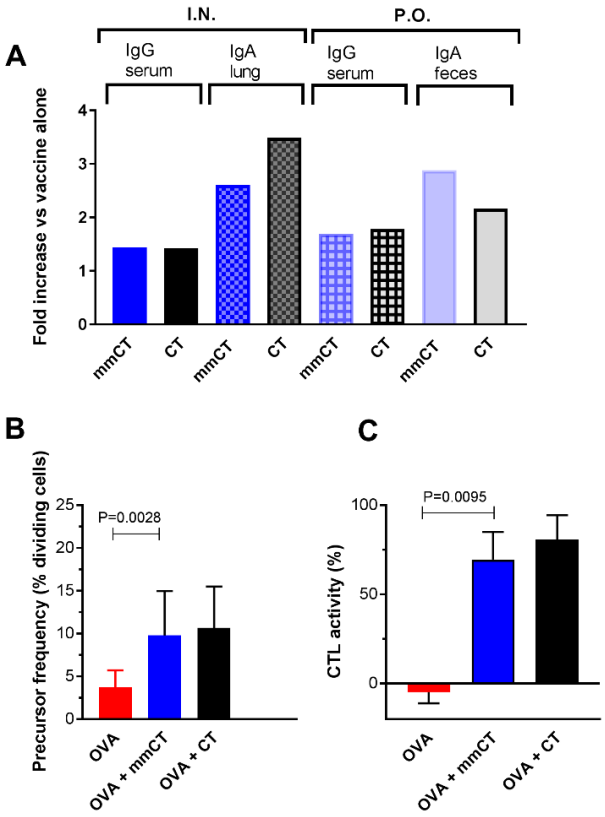

Figure 14. A) Adjuvant effect of mmCT and CT on serum and mucosal antibody responses to a model protein antigen after i.n. or p.o immunization. Data is expressed as the fold increase of anti-OVA isotype-specific antibody after immunization with vaccine plus mmCT or CT vs vaccine alone. B) Proliferation of antigen-specific CD4 T cells in draining lymph nodes in vivo after a single i.n. immunization with 2 µg OVA ±1.5 µg mmCT or CT. Mice were immunized one day after adoptive transfer of CFSE-labelled CD4 OT-II T cells, and three days later cervical lymph nodes (CLN) were collected and analyzed by flow cytometry, and the percentage of OT-II cells that had divided estimated. (C) CTL-activity in vivo after two i.n. immunizations two weeks apart with 50 µg OVA ±1.5 µg mmCT or CT, followed by i.v. injection of OVA peptide (SINFEKL)-pulsed target cells one week after the second immunization. Target cells were then collected from the spleen one day later and analyzed by flow cytometry, and the percentage of specifically killed target cells calculated as a measure of CTL activity. Data are from one of two experiments giving similar results.

mmCT ENHANCES IMMUNE RESPONSES TO MUCOSALLY ADMINISTERED CHOLERA AND INFLUENZA VACCINES

In order to test the effect of mmCT in the context of vaccine antigens, mice were co-administered mmCT either p.o. with a whole-cell cholera vaccine, or i.n. with influenza vaccine HA. In this case also, mmCT demonstrated strong adjuvant activity on intestinal anti-cholera LPS and the lung anti-HA mucosal IgA responses, respectively (Figure 15). Thus, mmCT despite its lack of enterotoxicity retains strong adjuvant activity for mucosally co-administered vaccine antigens.

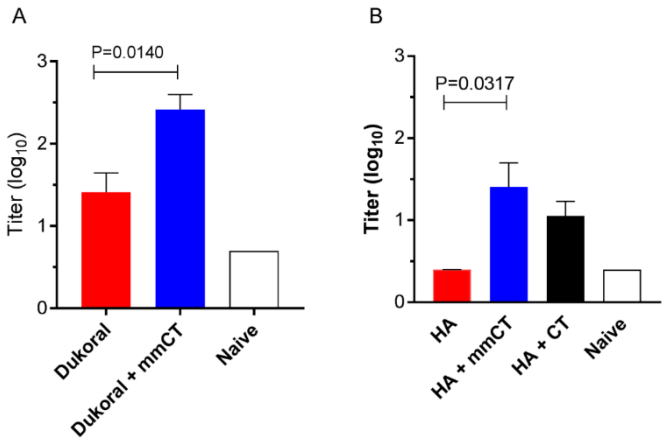

Figure 15. Adjuvant effect of mmCT on mucosal IgA antibody responses to mucosally co-administered Dukoral® cholera vaccine or influenza vaccine.

Overall, mmCT appears to be a promising adjuvant based on these pre-clinical studies for enhancing both mucosal and systemic humoral and cellular immune responses to co-administered mucosal vaccines and certainly warrants further investigations in the future in a clinical context both for human and veterinary vaccines.

PAPER III: IMPORTANT ROLE FOR THE NF-κB CLASSICAL PATHWAY IN THE ADJUVANT MECHANISM OF BOTH CT AND mmCT

NF-κB signaling occurring in APCs is an important component of the immune response. Such signaling usually occurs through the interaction between PAMPs and membrane-bound or cytosolic PRRs. NF-κB activation leads to its translocation into the cell nucleus, DNA binding and subsequent increased expression of cytokines, chemokines and adhesion molecules important for APC activation and induction of adaptive immune responses [142].

In order to better understand the mechanism through which CT exerts its effect, we investigated initially: A) whether CT induces nuclear translocation of NF-κB in APCs and if such induction leads to activation of downstream functional pro-inflammatory NF-κB signalling; B) whether this is mediated through a CT-induced activation of the cAMP-PKA pathway; and to which extent NF-κB signaling is responsible for CT's adjuvant effect.

Since CT is too toxic to be used as a vaccine adjuvant in humans, we also investigated the role of NF-κB for the adjuvant activity on human APCs and monocytes of mmCT.

NF-κB SIGNALING IS ACTIVATED BY CAMP-PKA SIGNALING IN RESPONSE TO mmCT

Using a human monocyte cell line THP1 with an NF-κB activation reporter system, we show that mmCT induced NFκB signaling in human monocytes. Our previous work had demonstrated that the predominant Th17-promoting adjuvant effect of mmCT on human immune cells *in vitro* is mediated via CT-induced cAMP-PKA signaling in monocytes and other APCs [107]. This was also confirmed in this study by treatment of THP1Blue−NF-κB cells with a PKA inhibitor prior to addition of mmCT which abolished the signal for NF-κB activation (figure 16).

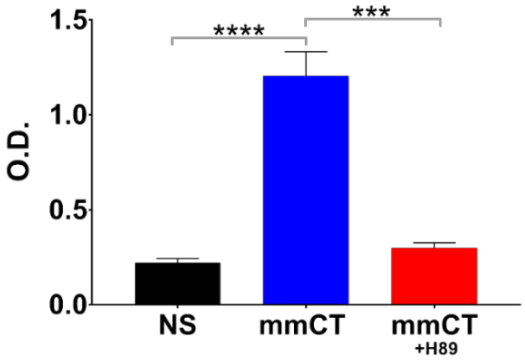

Figure 16. Human monocyte cell line (THP1Blue−NF-κB) were treated in triplicates with or without 1μg/ml mmCT (black and blue bars, respectively), or with the PKA inhibitor H-89 for 1 h prior to treatment with 1μg/ml mmCT for 16 h (red bar).

NF-κB SIGNALING IS ALSO REQUIRED FOR THE ADJUVANT ACTION OF mmCT ON HUMAN IMMUNE CELLS

Next, we examined whether NFκB signaling is required for the adjuvant action of mmCT on primary human APCs using a previously established co-culture system: purified human blood monocytes, were incubated with mmCT or medium, and then after thorough washing, the APCs were co-cultured with autologous $CD4^+$ T cells in the presence of SEB superantigen. Where after the levels of IL-17A, the predominant T cell cytokine increased by CT treatment of human APCs, were measured [107]. The advantage of using monocytes is that they are APC-precursors, well represented in PBMCs (≈10%) compared to DCs (1-2%), and that they have been shown to be able to induce $CD4^+$ T cell differentiation [143, 144].

In the present study, monocytes purified from human peripheral blood were either pre-treated with CAPE, a specific NFκB protein inhibitor, or left untreated, prior to the addition of mmCT or medium alone and the standard following procedures. The results show that while Th17 responses were significantly enhanced using mmCT-treated monocytes, the effect was effectively abrogated by pre-treating APCs with the specific NFκB inhibitor (figure 17).

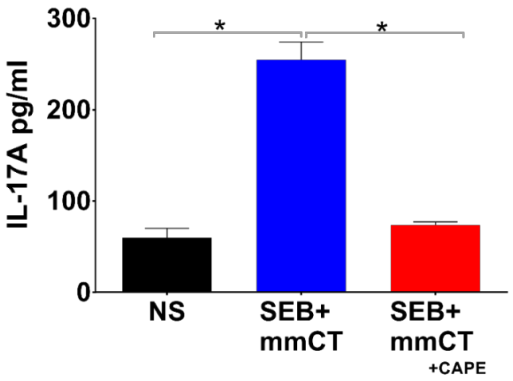

Figure 17. Purified CD14+ monocytes tested in triplicates were left untreated or were treated for 16 h with 1μg/ml of mmCT or in (E) with the NF-κB inhibitor CAPE for 1 h before the 16 h treatment with 1μg/ml mmCT. The cells were then co-cultured for 3 days with autologous CD4+ T cells plus SEB, and secreted IL-17A in culture supernatants determined. Bars represent means plus SEM of IL-17A concentrations in culture supernatants measured by ELISA

mmCT - INDUCED ACTIVATION OF NF-κB IN APCS PROMOTES IL-1 SIGNALING

CT and mmCT-driven production of IL-17 is itself known to be driven by IL-1 signaling from APCs. Inhibition of IL-1 signaling in human monocytes abrogates the Th17-promoting adjuvant effect of CT [107]. To investigate whether the mmCT driven stimulation of NF-κB also leads to IL-1 signaling in APCs, monocytes were treated with mmCT in presence or absence of the CAPE NF-κB inhibitor. Intracellular IL-1β expression was then measured by flow cytometry. Consistent with our previous findings, mmCT induced strong upregulation of IL-1β in human monocytes, which was almost completely abrogated in cells pre-treated with CAPE (figure 18). These findings demonstrate that the mmCT-induced increase in IL-1β signaling in APCs is strongly NFκB-dependent.

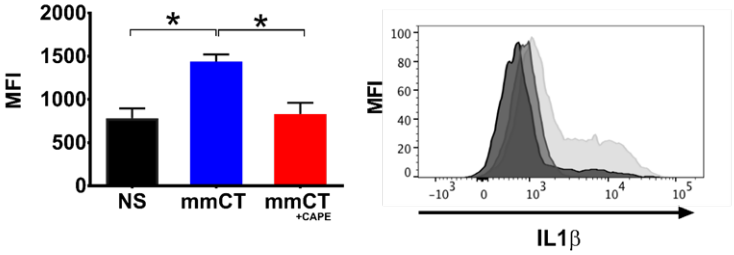

*Figure 18. Bar graphs representing MFI (A) and histogram overlays (B) of intracellular IL-1β expression in gated CD14+ monocytes treated either with mmCT (light grey filled histogram), with mmCT after preceding CAPE treatment (medium grey filled histogram), or with only medium (dark grey filled histogram).*represents $p < 0.05$ for the indicated comparisons. Data are from one of three separate experiments showing similar results.*

PAPER IV: THSB1 AND ITG1B INVOLVED IN THE ADJUVANT ACTIVITY IN CT AND mmCT

FUNCTIONAL ENRICHMENT ANALYSIS IDENTIFIES CT-INDUCED METABOLIC AND IMMUNE-RELATED PATHWAYS

Paper IV describes results from high-throughput proteomic analysis which allowed us to investigate global and differential expression of proteins in CT-treated human monocytes at 2 h, 4 h, 6 h and 16 h. Bioinformatics analyses allowed us to identify protein abundance, and major signaling pathways involved. Results demonstrate that three main biological pathways are activated among upregulated proteins peaking at 16 h: cellular organization, metabolism, and immune response (figure 19). The orchestrated induction of these sets of proteins by CT appears to characterize the shift from a steady "resting" state monocyte into a dynamic, energy-demanding and immunologically active APC. APC activation is known to be a metabolically demanding process, and requires efficient production and utilization of energy. Commonly used adjuvants have been found to induce a metabolic shift in APCs including activation of carbohydrate metabolism [145] and/or increased expression of mitochondrial proteins [146].

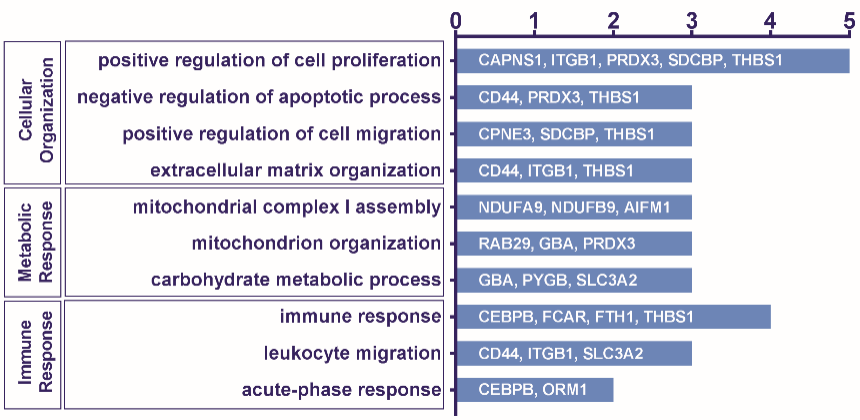

Figure 19. Functional enrichment analysis of upregulated proteins in CT-treated monocytes.

Specifically, amongst proteins classified under immune response category, we note a strong upregulation of thrombospondin-1(THBS1) and integrin beta-1(ITGB1) that we subsequently confirmed by quality control studies to test their expression using ELISA and FACS (figure 20). Using STRING database to identify relevant protein-protein interactions, we also noted a close association between THSB1 and ITGB1. For this reason we focused our further analysis on whether these proteins have a role in the adjuvant activity of CT and mmCT.

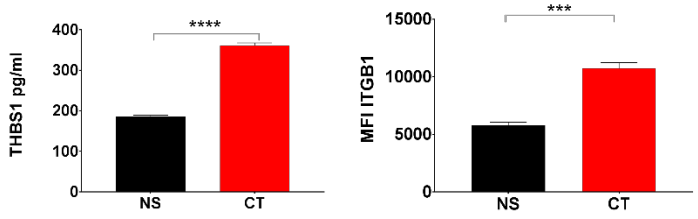

Figure 20. THBS1 and ITGB1 protein expression were measured by ELISA and flow cytometry, respectively, in monocytes incubated with CT or left untreated for 16 h. Bars represent mean plus SEM of THBS1 protein concentration in cell supernatants (A) or cell surface median fluorescence intensity (MFI) of ITGB1

INHIBITION OF THSB1 AND ITGB1 EXPRESSION IN APCS ABROGATES THE TH17 PROMOTING ADJUVANT EFFECT OF CT

We found that the Th17 responses were significantly reduced when THSB1 and ITGB1 in APCs were blocked by siRNAs treatments, indicating that THSB1 and ITGB1 play a role in responses in that lead to secretion of IL-1β and subsequent activation of T cells (figure 21).

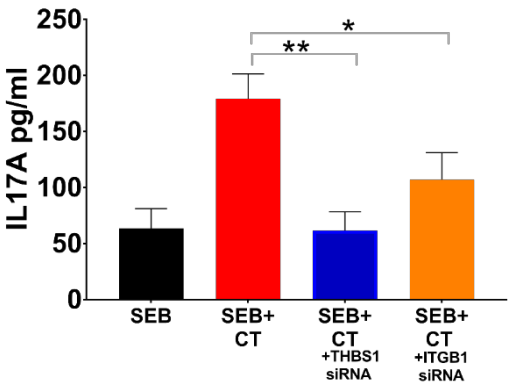

Figure 21. Purified CD14⁺ monocytes treated and non-treated for 24 h with siRNAs against THBS1 and ITGB and subsequently incubated for 16 h with CT, were co-cultured with autologous CD4⁺ T cells plus the polyclonal stimulus SEB for 3 days where after IL-17A cytokine levels were measured in supernatants by ELISA. Bars show mean and SEM of IL-17A cytokine levels

THE INDUCTION OF THSB1 AND ITGB1 IS DEPENDENT ON CAMP-PKA PATHWAY AND ON NF-κB SIGNALING

The blocking of cAMP production, or the inhibition of PKA activity or NF-κB signaling in APCs, were all found to abrogate the increased expression of THSB1 and ITGB1. We further showed that when we treated monocytes with siRNA specific for Guanine Nucleotide-Binding Protein G Subunit Alpha (GNAS), a key protein that activates adenylate cyclase. Treatment of monocytes with GNAS-specific siRNA resulted in reduced capacity of CT to induce expression of THSB1 or ITGB1 (figure 22). Thus CT driven expression of THSB1 and ITGB1 both seem to be dependent on cAMP driven activation of NFκB.

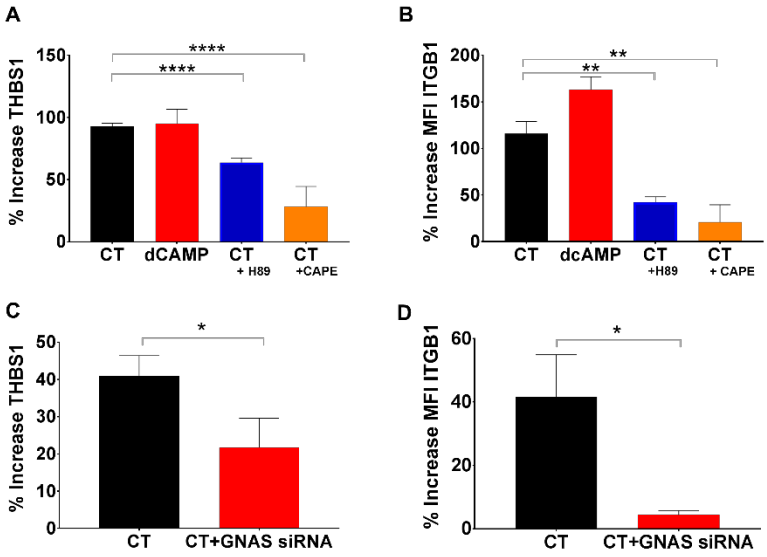

Figure 22. Purified CD14⁺ monocytes were left untreated or treated for 16 h with CT with the PKA inhibitor H-89 or with NF-κB inhibitor CAPE or the cAMP analogue dcAMP. In each case an untreated control was included. CD14⁺ monocytes were also left untreated or treated for 24 h with GNAS (Gsα)-specific siRNA, washed, and subsequently treated for 16 h with CT. Percentage differences in expression of THSB1 (A,C) or ITGB1 (B,D) in comparison with untreated cells are shown.

ADJUVANT EFFECTS OF mmCT ON HUMAN MONOCYTES ALSO REQUIRE THSB1 AND ITGB1.

Lastly, we show that the inhibition of either of these proteins by specific siRNAs strongly abrogated the adjuvant action of mmCT (figure 23) and suggests that despite the difference in cAMP production between mmCT and native CT, they appear to be acting through similar mechanisms dependent upon similar patterns of immune cell activation.

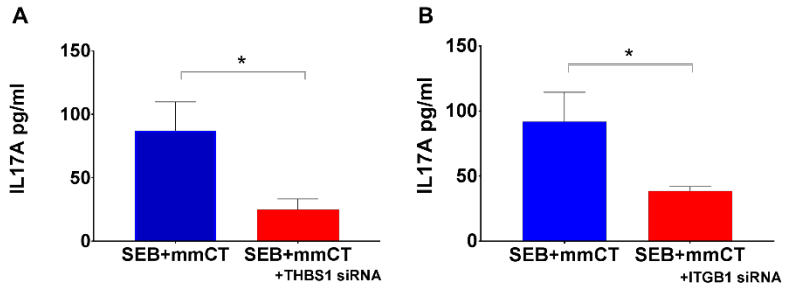

Figure 23. Purified CD14⁺ monocytes were incubated with siRNAs specific for THSB1 (A), ITGB1 (B) for 24 h. Cells were then left untreated or were treated for 16 h with mmCT, washed, and co-cultured for three days with autologous CD4⁺ T cells plus SEB. Bars represent mean plus SEM of IL-17A levels in culture supernatants measured by ELISA.

As discussed in paper III, the adjuvant effect of CT appears to be mainly cAMP/PKA/NF-κB-dependent. We show in this work that cAMP/PKA is also crucial for CT and mmCT-induced THSB1 and ITG1 expression. Thus, the promotion of Th17 responses by CT and mmCT requires at least a low level cAMP response in adjuvant-treated APCs, and is, at least as tested here *in vitro* using human APCs, dependent on immuno-modulatory proteins THSB1 and ITGB1.

The direct interaction between these two molecules has not been studied in this paper, and should be further investigated. However, while the present study points to a strong unidirectional immuno-stimulatory role of THSB1 and ITGB1 in APCs in response to CT and mmCT, several studies in *THSB1*$^{-/-}$ mice have suggested that THSB1 can exert both pro- and anti-inflammatory effects, with the latter proposed to serve as an immunoregulatory brake preventing excessive tissue [147] damage after immune stimulation. It remains to be defined to which extent the immunostimulatory effects of THSB1 and ITGB1 found in human APCs after treatment with CT and related adjuvants *in vitro* would be manifested also *in vivo*. It could be that in *THSB1*$^{-/-}$ mice, existing compensatory effects may mask a specialized immunostimulatory function of THSB1 in APCs. A way to address this issue in future studies could be to use Cre/*lox* APC-specific inducible knockout mice instead. Alternatively, the reported binding properties of THSB1 to multiple receptors [148], possibly in a concentration-dependent manner, might differentially modulate the balance in favour either of immune enhancement or down-regulation.

Altogether, we show that although mass spectrometry-based proteomics is associated with significant limitations [149], including limited ability to detect low abundance proteins such as inflammatory cytokines [150], it was able to identify proteins that were previously not associated with the adjuvant effect of CT and related non-toxic derivatives.

Our results suggest that as tested on human APCs and T cells *in vitro*, the adjuvant activity of CT and mmCT is regulated by THSB1 and ITGB1, and the adjuvant-induced expression of these proteins in human APCs similar to other observed adjuvant mechanisms by these agents is cAMP/PKA/NFκB-dependent.

5 GENERAL DISCUSSION

The first part of this thesis (paper I) focuses on vaccine manufacture processes and describes the construction of non-antibiotic strains able to produce high levels of recombinant proteins. Vaccines require uncomplicated, economical large-scale manufacturing processes since this will have long-term implications both for vaccine supply and cost. Furthermore, with the emergence of antibiotic resistance bacteria and the increased spread of antibiotics in the environment, the development of non-antibiotic selection systems for production of recombinant proteins in general is urgently needed. The *lgt*-based selection system allows maintenance of protein expression plasmids without the need for antibiotics. We demonstrate that the system is efficient, versatile and free from need for special growth media or temperatures inherent in most other antibiotic-free expression systems. Expression plasmids for the high-yield production of recombinant proteins using *lgt*-deleted strains of *E. coli* and *V. cholera* clearly show the potential of the system.

The selection method is still under development in our laboratory. The maintenance plasmid in the parental strain is based on a temperature-sensitive replicon with a single mutation, allowing the strain to only grow at 30°C and not at 39°C. However, we found a significant number of revertants are able to grow at non-permissive temperatures. This is not ideal because it complicates the selection of correct clones. Therefore, we will explore additional strategies for introducing expression vectors into the *lgt*-deleted background.

In addition to the production of recombinant proteins, the system can be used for a variety of applications that require stable maintenance of plasmids in the absence of antibiotic selection. It is possible for example, to create model pathogens, to study the activation of virulence factors using fluorescent markers. Furthermore, future work will be directed towards generating *lgt*-deleted strains for live-attenuated or killed whole cell vaccine using plasmids overexpressing vaccine antigens.

The second contribution towards this thesis was the construction of the highly promising non-toxic mucosal adjuvant, mmCT (Paper II). Despite the obvious appeal of using the *lgt* expression system to produce mmCT, such toxins tend to be toxic when over-produced. We therefore inserted the mutant *ctxA* gene

into the chromosome of a *V. cholera*e strain that naturally produces large amounts of toxin.
We demonstrate that mmCT lacks enterotoxicity, but largely retains the mucosal adjuvant activity of native CT in a number of *in vivo* and *in vitro* models. The enterotoxicity of CT is closely associated with increased levels of cAMP in the intestinal mucosa and the lack of enterotoxicity of mmCT most likely results from the dramatic reduction of cAMP production.

The lack of enterotoxicity of mmCT activity was expected since it was modelled on the dmLT protein developed by Clemens and co-workers [121] which was genetically engineered to be resistant to proteolytic cleavage between the A1 and A2 parts of the enzymatically active LT A subunit. As described earlier, this cleavage is known to be essential for the toxicity of both CT and LT.

It may be more surprising that both mmCT and dmLT can still induce some cAMP production despite being resistant to proteolytic cleavage. It is therefore still an unanswered question whether despite the complete resistance to proteolytic cleavage *in vitro*, there is still a low-level release of A1 from the A2-5B complex in the ER. This could explain the residual cAMP formation in mmCT or dmLT treated cells *in vivo*.

Alternatively, there might be a low-level release and "leakage" into the cytosol of the uncleaved toxin A subunit. Norton *et al*. [121] described that they could demonstrate uncleaved but rapidly degraded dmLTA (and no dmLTA1) in the cytosol of cells treated with dmLT. Moreover, they speculate that this rapid degradation (which was not seen with LT holotoxin) together with reduced ADP-ribosylating activity, is due to the presence of the LTA2, which interferes with LTA1/ARF interactions. They suggest such interference may contribute to the lack of dmLT toxicity and therefore explain the low-level residual cAMP inducing activity of dmLT. Whereas this may be the case, the rapid degradation of dmLT (and therefore probably of mmCT) could equally result from the uncleaved LT A subunits being unable to escape ubiquination in the ER. This would not allow them to refold in the cytosol.

A third possibility could be that the A subunits remain trapped in the endoplasmic reticulum (ER), and induce ADP-ribosylation of intermediate molecules that indirectly stimulate Gsα. It remains to be clarified how much

of the catalytic site is still active when masked by the CTA2 and what the intermediate mechanisms involved are.

Our previous work on human immune cells highlights that the primary action of adjuvants is on APCs. These studies have showed that when human APCs (monocytes and dendritic cells) are stimulated with CT (or mmCT), and co-cultured with autologous $CD4^+$ T cells, IL-17 responses by the $CD4^+$ T cells are strongly enhanced. Moreover, the adjuvant action of CT was found to be cAMP–PKA and caspase-1/ inflammasome dependent, and is mediated by IL-1β signaling [107]. Based on these studies, we extended investigations in mouse and human APCs and identified cAMP/PKA-dependent canonical NF-κB signaling as a key component in the adjuvant action of both CT and mmCT.

Despite this, it was not possible in these current thesis studies to elucidate the detailed molecular mechanisms by which mmCT and CT induce cAMP-PKA signaling and activate NFκB. Moreover, PKA is known to trigger nuclear translocation and increased transcriptional activity of NFκB by phosphorylation of RelA [151] and, although we could demonstrate the translocation of RelA in response to CT, we could not directly determine whether this was driven by phosphorylation.

Further, we found a novel an immuno-stimulatory role of THSB1 and ITGB1 in APCs. Both proteins were significantly overexpressed in response to CT treatment in a cAMP/PKA/NFκB-dependent manner. Preliminary results suggest that the same is true for mmCT since inhibition of THSB1 expression inhibited the production of IL-17 despite the finding that mmCT has lost most of the cAMP inducing activity of the native CT.

Future work should aim first to localize the mmCT A subunit (and/or the A1 fragment), in order to clarify whether or not the molecule remains trapped in Golgi, or ER or manages to access to the cytosol in an active form. Secondly, *in vitro* ADP-ribosyltransferase assays should be carried out looking at the interaction of mmCT with ARFs compared to CT. Moreover the levels of enzymatic activity retained by the mmCTA1 in presence of mmCTA2 should also be examined further. Another important experiment could also be to compare the ADP ribosylome of whole cells and even cell fractions in response to treatment with CT and mmCT. This could potentially answer the question of whether there are other targets for CT involved in its adjuvant action and

whether mmCT is able to escape the ER and ADP ribosylate Gsα in the plasma membrane.

An equally difficult question to answer is how mmCT, after having lost so much of the cAMP-inducing activity compared to CT can still retain so much adjuvant activity. Despite the cAMP levels induced by mmCT in APCs being only a fraction of those induced by CT, the amplification of the response in the signaling transduction pathway is similar in magnitude. From these findings, it seems that the little amount of cAMP induced by mmCT as stated before is "sufficient and necessary" to trigger the downstream signals needed for a strong adjuvant effect bearing in mind that the function of signaling pathways is to trigger molecular cascades to produce some larger outcome. It could also be that the very high levels of cAMP induced in APCs by CT are not optimal for the adjuvant action, and might even be negative. For instance, by exerting an anti-proliferative effect on both the APCs and on surrounding antigen-specific immune cells [152, 153].

Proteomics and *in vitro* studies carried out as part of this thesis also suggest an important role for two immunomodulatory proteins, THSB1 and ITGB1, in the adjuvant action of enterotoxin-based adjuvants. Whilst, these studies did not address the direct interaction between the two proteins, since inhibition of either of them was found to result in abrogation of the adjuvant effect, it could be postulated that the THSB1 interacting with the ITGB1 may provide an autocrine or paracrine loop that is important in the monocyte activation by CT. Evidence in the literature suggests that their interaction may lead to enhanced NF-κB/ inflammasome-dependent IL-1β production and maturation [154-157]. In the future, it would be interesting to clarify whether the observed THSB1-ITGB1 interaction influences inflammasome pathway activation and if so, in what way.

In conclusion, my PhD thesis work has generated two very different but equally promising tools in vaccine development; the *lgt* selection system for vaccine antigen manufacture and the novel mmCT adjuvant protein. Our work has also significantly contributed to a better understanding of the mechanisms of the adjuvant action of mmCT as well as native CT on APCs. Figure 24 summarizes these findings and the current understanding of the mechanisms of adjuvant action of CT and mmCT. Upon binding to its receptor (figure 24), CT as well as mmCT induce activation of the cAMP/PKA/classical NF-κB

signaling pathway. Then through NF-κB dependent stimulation of THSB1/ITGB1 complexes and/or other as yet unidentified NF-κB dependent molecules inflammasome/caspase 1 is activated. The activation of these pathways in turn induces expression and activation of cytokines and immunomodulatory proteins, which have been shown to be crucial for the adjuvant action in APCs with IL-1β being identified as a key secreted molecule in the increased activation of T cells, especially Th17 cells, by mmCT- and CT-stimulated human APCs.

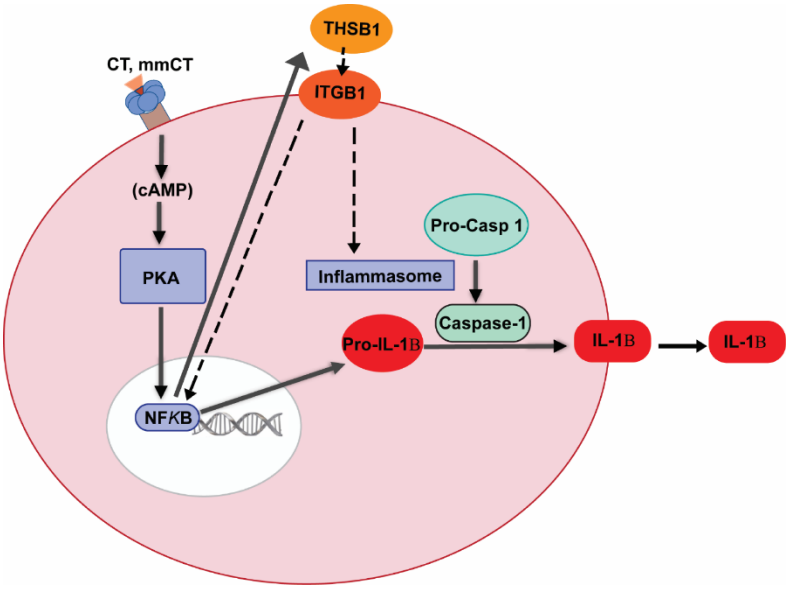

Figure 24. Mechanism of adjuvant action of CT and mmCT

The findings produced in this thesis will provide valuable novel insights in the vaccine/adjuvant area and in the mechanism of action of cAMP-inducing adjuvants, and may aid in the rational design of future vaccines.

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Ora e´ il momento dei ringraziamenti a chi e in Italia e in Italiano ☺.
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A tutti i miei **amici**, in particolare alle
mie gallinelle preferite con i loro pulcini e galletti,
Alla mia "**insidacabile famiglia**", "**cugine**" incluse,
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Ai miei **nipoti**, in particolare **Carlotta** e **Martina**,
A **nonno Francesco e ai nonni lassu´**,
A **Daniela** e **Francesca** con rispettivi,
A **mamma** e **papa´**,
dico immensamente **GRAZIE**!

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REFERENCES

1. Smith, K.A., *Edward jenner and the small pox vaccine.* Front Immunol, 2011. **2**: p. 21.
2. Greenwood, B., *The contribution of vaccination to global health: past, present and future.* Philos Trans R Soc Lond B Biol Sci, 2014. **369**(1645): p. 20130433.
3. Klugman, K.P. and S. Black, *Impact of existing vaccines in reducing antibiotic resistance: Primary and secondary effects.* Proc Natl Acad Sci U S A, 2018. **115**(51): p. 12896-12901.
4. Mignon, C., R. Sodoyer, and B. Werle, *Antibiotic-free selection in biotherapeutics: now and forever.* Pathogens, 2015. **4**(2): p. 157-81.
5. Holmgren, J. and C. Czerkinsky, *Mucosal immunity and vaccines.* Nat Med, 2005. **11**(4 Suppl): p. S45-53.
6. Lycke, N. and M. Bemark, *Mucosal adjuvants and long-term memory development with special focus on CTA1-DD and other ADP-ribosylating toxins.* Mucosal Immunol, 2010. **3**(6): p. 556-66.
7. Freytag, L.C. and J.D. Clements, *Mucosal adjuvants.* Vaccine, 2005. **23**(15): p. 1804-13.
8. Guy, B., *The perfect mix: recent progress in adjuvant research.* Nat Rev Microbiol, 2007. **5**(7): p. 505-17.
9. Sanchez, J. and J. Holmgren, *Recombinant system for overexpression of cholera toxin B subunit in Vibrio cholerae as a basis for vaccine development.* Proc Natl Acad Sci U S A, 1989. **86**(2): p. 481-5.
10. Freytag, L.C. and J.D. Clements, *Bacterial toxins as mucosal adjuvants.* Curr Top Microbiol Immunol, 1999. **236**: p. 215-36.
11. Morona, R., et al., *Construction of plasmid vectors with a non-antibiotic selection system based on the Escherichia coli thyA+ gene: application to cholera vaccine development.* Gene, 1991. **107**(1): p. 139-44.
12. Baquero, F., J.L. Martinez, and R. Canton, *Antibiotics and antibiotic resistance in water environments.* Curr Opin Biotechnol, 2008. **19**(3): p. 260-5.
13. Tuller, T., et al., *Association between translation efficiency and horizontal gene transfer within microbial communities.* Nucleic Acids Res, 2011. **39**(11): p. 4743-55.
14. Cooper, T.F. and J.A. Heinemann, *Postsegregational killing does not increase plasmid stability but acts to mediate the exclusion of competing plasmids.* Proc Natl Acad Sci U S A, 2000. **97**(23): p. 12643-8.
15. Cranenburgh, R.M., et al., *Escherichia coli strains that allow antibiotic-free plasmid selection and maintenance by repressor titration.* Nucleic Acids Res, 2001. **29**(5): p. E26.

16. Hagg, P., et al., *A host/plasmid system that is not dependent on antibiotics and antibiotic resistance genes for stable plasmid maintenance in Escherichia coli.* J Biotechnol, 2004. **111**(1): p. 17-30.
17. Lakhani, S., *Early clinical pathologists: Edward Jenner (1749-1823).* J Clin Pathol, 1992. **45**(9): p. 756-8.
18. Berche, P., *Louis Pasteur, from crystals of life to vaccination.* Clin Microbiol Infect, 2012. **18 Suppl 5**: p. 1-6.
19. Baicus, A., *History of polio vaccination.* World J Virol, 2012. **1**(4): p. 108-14.
20. Mestecky J, B.J., McGhee JR, Lamm ME, Strober W, Cebra JJ, et al. . , *Mucosal Immunology*. Publishers 2005., St. Louis MO: LWW
21. Otczyk, D.C. and A.W. Cripps, *Mucosal immunization: a realistic alternative.* Hum Vaccin, 2010. **6**(12): p. 978-1006.
22. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.
23. Geginat, J., et al., *Plasticity of human CD4 T cell subsets.* Front Immunol, 2014. **5**: p. 630.
24. Harris, N.L. and F. Ronchese, *The role of B7 costimulation in T-cell immunity.* Immunol Cell Biol, 1999. **77**(4): p. 304-11.
25. Kapsenberg, M.L., *Dendritic-cell control of pathogen-driven T-cell polarization.* Nat Rev Immunol, 2003. **3**(12): p. 984-93.
26. Yewdell, J.W. and S.M. Haeryfar, *Understanding presentation of viral antigens to CD8+ T cells in vivo: the key to rational vaccine design.* Annu Rev Immunol, 2005. **23**: p. 651-82.
27. Schenkel, J.M. and D. Masopust, *Tissue-resident memory T cells.* Immunity, 2014. **41**(6): p. 886-97.
28. Zens, K.D. and D.L. Farber, *Memory CD4 T cells in influenza.* Curr Top Microbiol Immunol, 2015. **386**: p. 399-421.
29. Robinson, H.L. and R.R. Amara, *T cell vaccines for microbial infections.* Nat Med, 2005. **11**(4 Suppl): p. S25-32.
30. Esser, M.T., et al., *Memory T cells and vaccines.* Vaccine, 2003. **21**(5-6): p. 419-30.
31. Parker, D.C., *T cell-dependent B cell activation.* Annu Rev Immunol, 1993. **11**: p. 331-60.
32. MacLennan, I.C., et al., *Extrafollicular antibody responses.* Immunol Rev, 2003. **194**: p. 8-18.
33. Weintraub, A., *Immunology of bacterial polysaccharide antigens.* Carbohydr Res, 2003. **338**(23): p. 2539-47.
34. Amanna, I.J. and M.K. Slifka, *Mechanisms that determine plasma cell lifespan and the duration of humoral immunity.* Immunol Rev, 2010. **236**: p. 125-38.
35. Goodnow, C.C., et al., *Control systems and decision making for antibody production.* Nat Immunol, 2010. **11**(8): p. 681-8.
36. Kometani, K. and T. Kurosaki, *Differentiation and maintenance of long-lived plasma cells.* Curr Opin Immunol, 2015. **33**: p. 64-9.

37. Leach, S., A. Lundgren, and A.M. Svennerholm, *Different kinetics of circulating antibody-secreting cell responses after primary and booster oral immunizations: a tool for assessing immunological memory.* Vaccine, 2013. **31**(30): p. 3035-8.
38. Kurosaki, T., K. Kometani, and W. Ise, *Memory B cells.* Nat Rev Immunol, 2015. **15**(3): p. 149-59.
39. Campbell, D.J., et al., *Targeting T cell responses by selective chemokine receptor expression.* Semin Immunol, 2003. **15**(5): p. 277-86.
40. Kunkel, E.J. and E.C. Butcher, *Plasma-cell homing.* Nat Rev Immunol, 2003. **3**(10): p. 822-9.
41. Mora, J.R., et al., *Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells.* Nature, 2003. **424**(6944): p. 88-93.
42. Hamann, A., et al., *Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo.* J Immunol, 1994. **152**(7): p. 3282-93.
43. Lycke, N., et al., *Lack of J chain inhibits the transport of gut IgA and abrogates the development of intestinal antitoxic protection.* J Immunol, 1999. **163**(2): p. 913-9.
44. Svanborg-Eden, C. and A.M. Svennerholm, *Secretory immunoglobulin A and G antibodies prevent adhesion of Escherichia coli to human urinary tract epithelial cells.* Infect Immun, 1978. **22**(3): p. 790-7.
45. Bemark, M., P. Boysen, and N.Y. Lycke, *Induction of gut IgA production through T cell-dependent and T cell-independent pathways.* Ann N Y Acad Sci, 2012. **1247**: p. 97-116.
46. Neutra, M.R. and P.A. Kozlowski, *Mucosal vaccines: the promise and the challenge.* Nat Rev Immunol, 2006. **6**(2): p. 148-58.
47. Khader, S.A., S.L. Gaffen, and J.K. Kolls, *Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa.* Mucosal Immunology, 2009. **2**(5): p. 403-411.
48. Cao, A.T., et al., *Th17 Cells Upregulate Polymeric Ig Receptor and Intestinal IgA and Contribute to Intestinal Homeostasis.* Journal of Immunology, 2012. **189**(9): p. 4666-4673.
49. Jan Holmgren, A.L., *Diarrhea Caused by Bacteria*, in *Plotkin's Vaccines (Seventh Edition), 2018*. 2018.
50. Srivastava, A., et al., *Mucosal vaccines: a paradigm shift in the development of mucosal adjuvants and delivery vehicles.* APMIS, 2015. **123**(4): p. 275-88.
51. Czerkinsky, C. and J. Holmgren, *Mucosal delivery routes for optimal immunization: targeting immunity to the right tissues.* Curr Top Microbiol Immunol, 2012. **354**: p. 1-18.
52. Manicassamy, S. and B. Pulendran, *Modulation of adaptive immunity with Toll-like receptors.* Semin Immunol, 2009. **21**(4): p. 185-93.
53. Lycke, N., *Recent progress in mucosal vaccine development: potential and limitations.* Nat Rev Immunol, 2012. **12**(8): p. 592-605.

54. Shin, S., et al., *Oral vaccines against cholera.* Clin Infect Dis, 2011. **52**(11): p. 1343-9.
55. Arvas, A., *Vaccination in patients with immunosuppression.* Turk Pediatri Ars, 2014. **49**(3): p. 181-5.
56. Moloney, P.J., *The Preparation and Testing of Diphtheria Toxoid (Anatoxine-Ramon).* Am J Public Health (N Y), 1926. **16**(12): p. 1208-10.
57. *Diphtheria vaccine.* Wkly Epidemiol Rec, 2006. **81**(3): p. 24-32.
58. *Tetanus vaccine.* Wkly Epidemiol Rec, 2006. **81**(20): p. 198-208.
59. Hutter, J. and B. Lepenies, *Carbohydrate-Based Vaccines: An Overview.* Methods Mol Biol, 2015. **1331**: p. 1-10.
60. Poolman, J. and R. Borrow, *Hyporesponsiveness and its clinical implications after vaccination with polysaccharide or glycoconjugate vaccines.* Expert Rev Vaccines, 2011. **10**(3): p. 307-22.
61. Pichichero, M.E., *Protein carriers of conjugate vaccines: characteristics, development, and clinical trials.* Hum Vaccin Immunother, 2013. **9**(12): p. 2505-23.
62. Plotkin, S., *History of vaccination.* Proc Natl Acad Sci U S A, 2014. **111**(34): p. 12283-7.
63. Holmgren, J. and A.M. Svennerholm, *Vaccines against mucosal infections.* Current Opinion in Immunology, 2012. **24**(3): p. 343-353.
64. Svennerholm, A.M., et al., *Antibody-Responses to Live and Killed Poliovirus Vaccines in the Milk of Pakistani and Swedish Women.* Journal of Infectious Diseases, 1981. **143**(5): p. 707-711.
65. Svennerholm, A.M. and J. Holmgren, *Synergistic protective effect in rabbits of immunization with Vibrio cholerae lipopolysaccharide and toxin/toxoid.* Infect Immun, 1976. **13**(3): p. 735-40.
66. Czerkinsky, C. and J. Holmgren, *Vaccines against enteric infections for the developing world.* Philos Trans R Soc Lond B Biol Sci, 2015. **370**(1671).
67. Ciabattini, A., et al., *Vaccination in the elderly: The challenge of immune changes with aging.* Semin Immunol, 2018. **40**: p. 83-94.
68. Siegrist, C.A., *Neonatal and early life vaccinology.* Vaccine, 2001. **19**(25-26): p. 3331-46.
69. Prendergast, A.J., *Malnutrition and vaccination in developing countries.* Philos Trans R Soc Lond B Biol Sci, 2015. **370**(1671).
70. Rytter, M.J., et al., *The immune system in children with malnutrition--a systematic review.* PLoS One, 2014. **9**(8): p. e105017.
71. Weiner, H.L., et al., *Oral tolerance.* Immunol Rev, 2011. **241**(1): p. 241-59.
72. Kau, A.L., et al., *Human nutrition, the gut microbiome and the immune system.* Nature, 2011. **474**(7351): p. 327-36.
73. Moore, S.E., et al., *Impact of nutritional status on antibody responses to different vaccines in undernourished Gambian children.* Acta Paediatr, 2003. **92**(2): p. 170-6.

74. Czerkinsky, C. and J. Holmgren, *Enteric vaccines for the developing world: a challenge for mucosal immunology.* Mucosal Immunol, 2009. **2**(4): p. 284-7.
75. Di Pasquale, A., et al., *Vaccine Adjuvants: from 1920 to 2015 and Beyond.* Vaccines (Basel), 2015. **3**(2): p. 320-43.
76. Ramon, G., *[Certain works presented at the Academie Nationale de Medecine (Paris) from 1925 to 1950]*. Rev Immunol Ther Antimicrob, 1959. **23**: p. 359-401.
77. Reed, S.G., M.T. Orr, and C.B. Fox, *Key roles of adjuvants in modern vaccines.* Nature medicine, 2013. **19**(12): p. 1597-1608.
78. Savelkoul, H.F., et al., *Choice and Design of Adjuvants for Parenteral and Mucosal Vaccines.* Vaccines (Basel), 2015. **3**(1): p. 148-71.
79. Li, Z.W., et al., *The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis.* J Exp Med, 1999. **189**(11): p. 1839-45.
80. Hayden, M.S. and S. Ghosh, *Signaling to NF-kappaB.* Genes Dev, 2004. **18**(18): p. 2195-224.
81. Kawai, T. and S. Akira, *Signaling to NF-kappaB by Toll-like receptors.* Trends Mol Med, 2007. **13**(11): p. 460-9.
82. Shaw, P.J., M. Lamkanfi, and T.D. Kanneganti, *NOD-like receptor (NLR) signaling beyond the inflammasome.* Eur J Immunol, 2010. **40**(3): p. 624-7.
83. Huang, C.F., et al., *Effect of neonatal sublingual vaccination with native or denatured ovalbumin and adjuvant CpG or cholera toxin on systemic and mucosal immunity in mice.* Scandinavian Journal of Immunology, 2008. **68**(5): p. 502-510.
84. Casella, C.R. and T.C. Mitchell, *Putting endotoxin to work for us: Monophosphoryl lipid A as a safe and effective vaccine adjuvant.* Cellular and Molecular Life Sciences, 2008. **65**(20): p. 3231-3240.
85. Seubert, A., et al., *Adjuvanticity of the oil-in-water emulsion MF59 is independent of Nlrp3 inflammasome but requires the adaptor protein MyD88.* Proc Natl Acad Sci U S A, 2011. **108**(27): p. 11169-74.
86. De Gregorio, E., E. Tritto, and R. Rappuoli, *Alum adjuvanticity: unraveling a century old mystery.* Eur J Immunol, 2008. **38**(8): p. 2068-71.
87. Holmgren, J., I. Lonnroth, and L. Svennerholm, *Tissue receptor for cholera exotoxin: postulated structure from studies with GM1 ganglioside and related glycolipids.* Infect Immun, 1973. **8**(2): p. 208-14.
88. Cervin, J., et al., *GM1 ganglioside-independent intoxication by Cholera toxin.* PLoS Pathog, 2018. **14**(2): p. e1006862.
89. Backstrom, M., et al., *Structural basis for differential receptor binding of cholera and Escherichia coli heat-labile toxins: influence of heterologous amino acid substitutions in the cholera B-subunit.* Mol Microbiol, 1997. **24**(3): p. 489-97.

90. Sanchez, J. and J. Holmgren, *Cholera toxin structure, gene regulation and pathophysiological and immunological aspects.* Cell Mol Life Sci, 2008. **65**(9): p. 1347-60.
91. Clemens, J.D., et al., *Cholera.* Lancet, 2017. **390**(10101): p. 1539-1549.
92. Sanchez, J. and J. Holmgren, *Cholera toxin - a foe & a friend.* Indian J Med Res, 2011. **133**: p. 153-63.
93. Holmgren, J., *Actions of cholera toxin and the prevention and treatment of cholera.* Nature, 1981. **292**(5822): p. 413-17.
94. Lycke, N. and C. Lebrero-Fernandez, *ADP-ribosylating enterotoxins as vaccine adjuvants.* Curr Opin Pharmacol, 2018. **41**: p. 42-51.
95. Bromander, A.K., et al., *Cholera toxin enhances antigen presentation.* Adv Exp Med Biol, 1995. **371B**: p. 1501-6.
96. Bourque, D.L., et al., *Analysis of the Human Mucosal Response to Cholera Reveals Sustained Activation of Innate Immune Signaling Pathways.* Infect Immun, 2018. **86**(2).
97. Cong, Y., A.O. Oliver, and C.O. Elson, *Effects of cholera toxin on macrophage production of co-stimulatory cytokines.* Eur J Immunol, 2001. **31**(1): p. 64-71.
98. Gagliardi, M.C., et al., *Cholera toxin induces maturation of human dendritic cells and licences them for Th2 priming.* Eur J Immunol, 2000. **30**(8): p. 2394-403.
99. Mattsson, J., et al., *Cholera toxin adjuvant promotes a balanced Th1/Th2/Th17 response independently of IL-12 and IL-17 by acting on Gsalpha in CD11b(+) DCs.* Mucosal Immunol, 2015. **8**(4): p. 815-27.
100. Fahlen-Yrlid, L., et al., *CD11c(high) Dendritic Cells Are Essential for Activation of CD4(+) T Cells and Generation of Specific Antibodies following Mucosal Immunization.* Journal of Immunology, 2009. **183**(8): p. 5032-5041.
101. Eriksson, K., et al., *Cholera toxin and its B subunit promote dendritic cell vaccination with different influences on Th1 and Th2 development.* Infection and Immunity, 2003. **71**(4): p. 1740-1747.
102. Gustafsson, T., et al., *Direct interaction between cholera toxin and dendritic cells is required for oral adjuvant activity.* European Journal of Immunology, 2013. **43**(7): p. 1779-1788.
103. Grdic, D., et al., *Splenic marginal zone dendritic cells mediate the cholera toxin adjuvant effect: dependence on the ADP-ribosyltransferase activity of the holotoxin.* J Immunol, 2005. **175**(8): p. 5192-202.
104. Datta, S.K., et al., *Mucosal adjuvant activity of cholera toxin requires Th17 cells and protects against inhalation anthrax.* Proc Natl Acad Sci U S A, 2010. **107**(23): p. 10638-43.
105. Hirota, K., et al., *Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses.* Nat Immunol, 2013. **14**(4): p. 372-9.

106. Bromander, A., J. Holmgren, and N. Lycke, *Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro.* J Immunol, 1991. **146**(9): p. 2908-14.
107. Larena, M., et al., *Cholera toxin, and the related nontoxic adjuvants mmCT and dmLT, promote human Th17 responses via cyclic AMP-protein kinase A and inflammasome-dependent IL-1 signaling.* J Immunol, 2015. **194**(8): p. 3829-39.
108. Li, H.F., S. Nookala, and F. Re, *Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1 beta and IL-18 release.* Journal of Immunology, 2007. **178**(8): p. 5271-5276.
109. Schnare, M., et al., *Toll-like receptors control activation of adaptive immune responses.* Nat Immunol, 2001. **2**(10): p. 947-50.
110. Lambrecht, B.N., et al., *Mechanism of action of clinically approved adjuvants.* Curr Opin Immunol, 2009. **21**(1): p. 23-9.
111. Jackson, R.J., et al., *Optimizing Oral Vaccines - Induction of Systemic and Mucosal B-Cell and Antibody-Responses to Tetanus Toxoid by Use of Cholera-Toxin as an Adjuvant.* Infection and Immunity, 1993. **61**(10): p. 4272-4279.
112. Raghavan, S., A.M. Svennerholm, and J. Holmgren, *Effects of oral vaccination and immunomodulation by cholera toxin on experimental Helicobacter pylori infection, reinfection, and gastritis.* Infection and Immunity, 2002. **70**(8): p. 4621-4627.
113. Levine, M.M., et al., *New Knowledge on Pathogenesis of Bacterial Enteric Infections as Applied to Vaccine Development.* Microbiological Reviews, 1983. **47**(4): p. 510-550.
114. Mutsch, M., et al., *Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland.* N Engl J Med, 2004. **350**(9): p. 896-903.
115. Douce, G., et al., *Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin.* Infect Immun, 1997. **65**(7): p. 2821-8.
116. Stephenson, I., et al., *Phase I evaluation of intranasal trivalent inactivated influenza vaccine with nontoxigenic Escherichia coli enterotoxin and novel biovector as mucosal adjuvants, using adult volunteers.* J Virol, 2006. **80**(10): p. 4962-70.
117. Lewis, D.J., et al., *Transient facial nerve paralysis (Bell's palsy) following intranasal delivery of a genetically detoxified mutant of Escherichia coli heat labile toxin.* PLoS One, 2009. **4**(9): p. e6999.
118. Agren, L.C., et al., *Adjuvanticity of the cholera toxin A1-based gene fusion protein, CTA1-DD, is critically dependent on the ADP-ribosyltransferase and Ig-binding activity.* J Immunol, 1999. **162**(4): p. 2432-40.
119. Agren, L., et al., *The ADP-ribosylating CTA1-DD adjuvant enhances T cell-dependent and independent responses by direct action on B cells*

involving anti-apoptotic Bcl-2- and germinal center-promoting effects. J Immunol, 2000. **164**(12): p. 6276-86.
120. Mattsson, J., et al., *Complement activation and complement receptors on follicular dendritic cells are critical for the function of a targeted adjuvant.* J Immunol, 2011. **187**(7): p. 3641-52.
121. Norton, E.B., et al., *Characterization of a mutant Escherichia coli heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant.* Clin Vaccine Immunol, 2011. **18**(4): p. 546-51.
122. Summerton, N.A., et al., *Toward the development of a stable, freeze-dried formulation of Helicobacter pylori killed whole cell vaccine adjuvanted with a novel mutant of Escherichia coli heat-labile toxin.* Vaccine, 2010. **28**(5): p. 1404-11.
123. Lundgren, A., et al., *Safety and immunogenicity of an improved oral inactivated multivalent enterotoxigenic Escherichia coli (ETEC) vaccine administered alone and together with dmLT adjuvant in a double-blind, randomized, placebo-controlled Phase I study.* Vaccine, 2014. **32**(52): p. 7077-84.
124. Lee, Y.S., et al., *An alpha-GalCer analogue with branched acyl chain enhances protective immune responses in a nasal influenza vaccine.* Vaccine, 2011. **29**(3): p. 417-425.
125. Youn, H.J., et al., *A single intranasal immunization with inactivated influenza virus and alpha-galactosylceramide induces long-term protective immunity without redirecting antigen to the central nervous system.* Vaccine, 2007. **25**(28): p. 5189-5198.
126. Courtney, A.N., et al., *Alpha-galactosylceramide is an effective mucosal adjuvant for repeated intranasal or oral delivery of HIV peptide antigens.* Vaccine, 2009. **27**(25-26): p. 3335-3341.
127. Zygmunt, B.M., S.F. Weissmann, and C.A. Guzman, *NKT Cell Stimulation with alpha-Galactosylceramide Results in a Block of Th17 Differentiation after Intranasal Immunization in Mice.* Plos One, 2012. **7**(1).
128. Davitt, C.J.H., et al., *Alpha-galactosylceramide enhances mucosal immunity to oral whole-cell cholera vaccines.* Mucosal Immunology, 2019. **12**(4): p. 1055-1064.
129. Davitt, C.J., et al., *A novel adjuvanted capsule based strategy for oral vaccination against infectious diarrhoeal pathogens.* J Control Release, 2016. **233**: p. 162-73.
130. Chadwick, S., C. Kriegel, and M. Amiji, *Nanotechnology solutions for mucosal immunization.* Adv Drug Deliv Rev, 2010. **62**(4-5): p. 394-407.
131. Jonson, G., J. Sanchez, and A.M. Svennerholm, *Expression and detection of different biotype-associated cell-bound haemagglutinins of Vibrio cholerae O1.* J Gen Microbiol, 1989. **135**(1): p. 111-20.

132. Lebens, M., et al., *Construction of novel vaccine strains of Vibrio cholerae co-expressing the Inaba and Ogawa serotype antigens.* Vaccine, 2011. **29**(43): p. 7505-13.
133. Frisk, A., et al., *The role of different protein components from the Haemophilus ducreyi cytolethal distending toxin in the generation of cell toxicity.* Microb Pathog, 2001. **30**(6): p. 313-24.
134. Borde, A., et al., *Preparation and evaluation of a freeze-dried oral killed cholera vaccine formulation.* Eur J Pharm Biopharm, 2011. **79**(3): p. 508-18.
135. Svennerholm, A.M. and G. Wiklund, *Rapid GM1-enzyme-linked immunosorbent assay with visual reading for identification of Escherichia coli heat-labile enterotoxin.* J Clin Microbiol, 1983. **17**(4): p. 596-600.
136. Svennerholm, A.M., et al., *Monoclonal antibodies to Escherichia coli heat-labile enterotoxins: neutralising activity and differentiation of human and porcine LTs and cholera toxin.* Med Biol, 1986. **64**(1): p. 23-30.
137. Karlsson, S.L., et al., *Development of Stable Vibrio cholerae O1 Hikojima Type Vaccine Strains Co–Expressing the Inaba and Ogawa Lipopolysaccharide Antigens.* PLoS ONE, 2014. **9**(11): p. e108521.
138. Robertson, J.M., P.E. Jensen, and B.D. Evavold, *DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope.* J Immunol, 2000. **164**(9): p. 4706-12.
139. Fahlen-Yrlid, L., et al., *CD11c (high) dendritic cells are essential for activation of CD4+ T cells and generation of specific antibodies following mucosal immunization.* J Immunol, 2009. **183**(8): p. 5032-41.
140. Baselski, V., R. Briggs, and C. Parker, *Intestinal fluid accumulation induced by oral challenge with Vibrio cholerae or cholera toxin in infant mice.* Infect Immun, 1977. **15**(3): p. 704-12.
141. Smith, D.B. and K.S. Johnson, *Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase.* Gene, 1988. **67**(1): p. 31-40.
142. Hayden, M.S., A.P. West, and S. Ghosh, *NF-kappaB and the immune response.* Oncogene, 2006. **25**(51): p. 6758-80.
143. Acosta-Rodriguez, E.V., et al., *Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells.* Nat Immunol, 2007. **8**(6): p. 639-46.
144. Leach, S., et al., *The adjuvant double mutant Escherichia coli heat labile toxin enhances IL-17A production in human T cells specific for bacterial vaccine antigens.* PLoS One, 2012. **7**(12): p. e51718.
145. Everts, B. and E.J. Pearce, *Metabolic control of dendritic cell activation and function: recent advances and clinical implications.* Front Immunol, 2014. **5**: p. 203.

146. Krawczyk, C.M., et al., *Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation.* Blood, 2010. **115**(23): p. 4742-9.
147. Lopez-Dee, Z., K. Pidcock, and L.S. Gutierrez, *Thrombospondin-1: multiple paths to inflammation.* Mediators Inflamm, 2011. **2011**: p. 296069.
148. Resovi, A., et al., *Current understanding of the thrombospondin-1 interactome.* Matrix Biol, 2014. **37**: p. 83-91.
149. Kocher, T. and G. Superti-Furga, *Mass spectrometry-based functional proteomics: from molecular machines to protein networks.* Nat Methods, 2007. **4**(10): p. 807-15.
150. Stenken, J.A. and A.J. Poschenrieder, *Bioanalytical chemistry of cytokines--a review.* Anal Chim Acta, 2015. **853**: p. 95-115.
151. Zhong, H., R.E. Voll, and S. Ghosh, *Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300.* Mol Cell, 1998. **1**(5): p. 661-71.
152. Holmgren, J., N. Lycke, and C. Czerkinsky, *Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems.* Vaccine, 1993. **11**(12): p. 1179-84.
153. Holmgren, J., L. Lindholm, and I. Lonnroth, *Interaction of cholera toxin and toxin derivatives with lymphocytes. I. Binding properties and interference with lectin-induced cellular stimulation.* J Exp Med, 1974. **139**(4): p. 801-19.
154. Calzada, M.J., et al., *Alpha4beta1 integrin mediates selective endothelial cell responses to thrombospondins 1 and 2 in vitro and modulates angiogenesis in vivo.* Circ Res, 2004. **94**(4): p. 462-70.
155. Calzada, M.J., et al., *Identification of novel beta1 integrin binding sites in the type 1 and type 2 repeats of thrombospondin-1.* J Biol Chem, 2004. **279**(40): p. 41734-43.
156. Stein, E.V., et al., *Secreted Thrombospondin-1 Regulates Macrophage Interleukin-1beta Production and Activation through CD47.* Sci Rep, 2016. **6**: p. 19684.
157. Klein, S., et al., *Alpha 5 beta 1 integrin activates an NF-kappa B-dependent program of gene expression important for angiogenesis and inflammation.* Mol Cell Biol, 2002. **22**(16): p. 5912-22.