Journal of Controlled Release 233 (2016) 162–173

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

A novel adjuvanted capsule based strategy for oral vaccination against infectious diarrhoeal pathogens

Christopher J.H. Davitt [a,1], Edel A. McNeela [a,1], Stephanie Longet [a,1], Joshua Tobias [b], Vincenzo Aversa [c], Craig P. McEntee [a], Monica Rosa [c], Ivan S. Coulter [c], Jan Holmgren [b], Ed C. Lavelle [a,d,*]

[a] Adjuvant Research Group, School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, D02 PN40, Ireland
[b] University of Gothenburg Vaccine Research Institute (GUVAX), Dept. of Microbiology and Immunology, University of Gothenburg, Box 435, 405 30 Gothenburg, Sweden
[c] Sigmoid Pharma Limited, Dublin City University, The Invent Centre, DCU, Glasnevin, Dublin 9, Ireland
[d] Centre for Research on Adaptive Nanostructures and Nanodevices (CRANN); Advanced Materials Bio-Engineering Research Centre (AMBER), Trinity College Dublin, Dublin 2, D02 PN40, Ireland

ARTICLE INFO

Article history:
Received 13 February 2016
Received in revised form 29 April 2016
Accepted 1 May 2016
Available online 5 May 2016

Chemical compounds studied in this article:
Alpha-galactosylceramide (KRN 7000) (PubChem CID: 2826713)
Sorbitol (PubChem CID: 5780)
Kolliphor HS 15 (Solutol® HS 15) (PubChem CID: 71311956)
Eudragit L30D 55 (Alcogum) (PubChem CID: 107665)

Keywords:
Vaccine
Oral delivery
Adjuvant
Traveller's diarrhoea
Enteric
Capsule

ABSTRACT

Diarrhoeal infections are a major cause of morbidity and mortality with enterotoxigenic *Escherichia coli* (ETEC) and cholera imposing a significant global burden. There is currently no licensed vaccine for ETEC. Development of new nonliving oral vaccines has proven difficult due to the physicochemical and immunological challenges associated with the oral route. This demands innovative delivery solutions to protect antigens, control their release and build in immune-stimulatory activity. We describe the Single Multiple Pill® (SmPill®) vaccine formulation which combines the benefits of enteric polymer coating to protect against low gastric pH, a dispersed phase to control release and aid the solubility of non-polar components and an optimized combination of adjuvant and antigen to promote mucosal immunity. We demonstrate the effectiveness of this system with whole cell killed *E. coli* overexpressing colonization factor antigen I (CFA/I), JT-49. Alpha-galactosylceramide was identified as a potent adjuvant within SmPill® that enhanced the immunogenicity of JT-49. The bacteria associated with the dispersed phase were retained within the capsules at gastric pH but released at intestinal pH. Vaccination with an optimized SmPill® formulation promoted CFA/I-specific immunoglobulin A (IgA) responses in the intestinal mucosa in addition to serum IgG and a solubilized adjuvant was indispensable for efficacy.

1. Introduction

Infectious diarrhoea is a highly prevalent cause of morbidity and mortality in developed and developing countries. Children and infants are most susceptible to diarrhoeal infections, accounting for over 600,000 deaths and millions of hospitalizations annually, concentrated mostly in low income countries [1,2]. Despite the availability of treatments for diarrhoeal infections, such as oral rehydration therapy and antibiotics, repeated bouts of infection during childhood are common. Moreover, these episodes of diarrhoea have been correlated to lasting intestinal damage and may result in malnutrition and impaired development in adult life, perpetuating cycles of poverty in these regions [3]. One of the most prevalent global causes of enteric diarrhoeal death in children, and other demographics is enterotoxigenic *Escherichia coli* (ETEC) [4]. ETEC is primarily spread via the faecal-oral route, most commonly through contaminated water and food. ETEC is largely found in freshwater bodies such as rivers and reservoirs, with

Abbreviations: AUC, area under the curve; BSA, bovine serum albumin; CF, colonization factor; CFA, colonization factor antigen; CT, cholera toxin; EDTA, ethylenediaminetetraacetic acid; ETEC, enterotoxigenic *Escherichia coli*; FITC, fluorescein isothiocyanate; GEMS, Global Enterics Multicenter Study; GFP, green fluorescent protein; GIT, gastrointestinal tract; IEC, intestinal epithelial cell; iNKT, invariant natural killer T cell; JT-227, CFA/I-positive ETEC strain expressing GFP; JT-49, *E. coli* K12 strain overexpressing CFA/I; LT, heat-labile toxin of ETEC; mAb, monoclonal antibody; OEV, oral ETEC vaccine; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SIgA, secretory immunoglobulin A; SmPill®, Single Multiple Pill®; STI, soybean trypsin inhibitor; WCK, whole cell killed; WT, wild type; α-Galcer, alpha-galactosylceramide.

* Corresponding author at: Adjuvant Research Group, School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, D02 PN40, Ireland.
E-mail address: lavellee@tcd.ie (E.C. Lavelle).
[1] Authors made equal contributions to the research in this manuscript.

http://dx.doi.org/10.1016/j.jconrel.2016.05.001
0168-3659/

infections being most prevalent in spring and summer [5]. Although ETEC is encountered globally, it is primarily concentrated in Sub-Saharan Africa and South Asia and recent reports from the Global Enterics Multicenter Study (GEMS) suggests that ETEC will remain in the top four pathogens causing childhood diarrhoea in these regions [6]. Moreover, ETEC is also a major health concern for aid workers, military personnel and tourists visiting affected regions and is the leading cause of traveller's diarrhoea in westerners [7]. Although traveller's diarrhoea is an acute, short term infection, some cases can progress to a more severe and chronic condition such as idiopathic inflammatory bowel disease leading to life-long consequences [8,9]. In addition, increased global travel by westerners makes the development of an effective vaccine against ETEC a priority [10].

Following ingestion, ETEC bacteria do not invade, but rather colonize the small intestine by adherence to intestinal epithelial cells (IECs) via fimbrial colonization factors (CFs) [11], of which 25 have been identified in different strains of ETEC [11]. However, the most commonly detected CF antigens (CFAs) are CFA/I, CS1, CS2, CS3, CS4, CS5 and CS6. Pathogenesis is associated with the delivery of heat-labile (LT) and/or heat-stable toxins, both leading to secretory diarrhoea. Victims may die of dehydration if adequate fluid and electrolyte replacement therapy cannot be accessed in time [12]. Toxin delivery is exclusively mediated through intimate contact with IECs and is considered a key pathogenic event, while blocking this process prevents the bacterium from exerting its pathogenic effects. Rabbit models have demonstrated that local antibody responses directed against CFAs play a key role in protective immunity [13]. Furthermore, natural immunity following repeated exposure to local ETEC strains expressing homologous CFAs is well documented in humans thus making CFAs promising antigenic targets for a candidate ETEC vaccine [14].

Oral vaccination has been demonstrated as the most effective method of inducing immune responses in the gut against enteric infections via the induction of secretory immunoglobulin A (SIgA). This was illustrated by the failure of injectable cholera vaccines to elicit satisfactory protection during trials in the 1960s [15,16], and the success of current oral cholera vaccines such as Dukoral® in large scale field trials [17]. Additionally, the introduction of oral rotavirus vaccination programs in endemic countries has had a very positive effect on disease burdens in these regions [18]. There is little doubt that the introduction of ETEC vaccination into these regions would have a further positive effect on the reduction of childhood diarrhoea cases. In contrast, early candidate oral ETEC vaccines (OEVs) failed to elicit satisfactory results in children from endemic regions [19]. Problems associated with poor immunogenicity in endemic populations are not uncommon as many prototype oral vaccines that are immunogenic in Western volunteers subsequently failed in field trials [16]. This may be due to the predisposition of gut immune responses to skew towards hypo-responsiveness and tolerance [20], in addition to differences in nutrition (often a lack of key nutrients e.g. Vitamin A), microbiota composition, previous natural exposure to antigen due to infection and the presence of helminths, which are often referred to collectively as "environmental enteropathy". Overcoming this challenge will be key to the future success of any OEV prototype for which there is an urgent global need [2].

One strategy to overcome the poor immunogenicity of oral vaccines is to increase the antigen load on whole cell killed (WCK) bacterial cells rather than increasing the dose of bacteria. Large doses of WCK bacteria have been reported to induce vomiting in children following oral administration [21]. Tobias et al. generated a non-toxic, formalin killed *E. coli* K12 strain overexpressing CFA/I (JT-49) [22], which has been shown to be one of the most conserved CFAs globally [23]. JT-49 exhibited enhanced surface expression of CFA/I compared to a wild type (WT) reference ETEC strain, allowing for a reduced bacterial dose to be administered while preserving antigenicity [22]. This also translated into enhanced immunogenicity in pre-clinical studies [24]. Furthermore this strain was safe, well tolerated and immunogenic during trials in Swedish volunteers [25]. The combination of enhanced immunogenicity over WT ETEC strains and positive outcomes in early human trials led us to choose JT-49 as a candidate antigen.

One of the biggest challenges of the oral route lies in overcoming the acidic pH of the stomach and the proteolytic environment of the duodenum, which can degrade labile components prior to their arrival at the key antigen sampling sites located in the gut [26]. These anatomical locations include the jejunum and ileum, both of which are rich in Peyer's patches (PPs) (PP-associated microfold cells being one of the key sites of antigen sampling in the GIT) and mucosal dendritic cells which play a key role in the elicitation of gut immune responses [27]. Therefore, oral vaccine delivery vehicles are attractive as a means to not only protect and target antigens, facilitate controlled release [26], and simplify delivery but also enhance the efficacy of mucosal vaccine formulations [28]. While many oral vaccine delivery systems have been evaluated in animal models, this has yet to be translated into efficacy in humans. In a phase one trial utilizing a microparticle-based oral delivery system containing purified ETEC CFAs, only 24% of subjects displayed an increase in antigen-specific serum IgA demonstrating that delivery alone may be insufficient at driving oral vaccine efficacy [29].

We hypothesized that an integrated system comprising a capsular delivery technology with an enteric (low pH-resistant) coating and an intrinsic controlled release mechanism, would enhance the efficacy of an immunogenic antigen in combination with an efficacious and safe adjuvant [26]. We describe the design of a novel Single-Multiple Pill® (SmPill®) vaccine formulation which combines these attributes. We previously reported a SmPill® oral drug delivery system [30,31], but there are demanding and unique challenges for the delivery of oral vaccines compared to small molecule drugs. These challenges include the marked susceptibility of protein antigens to degradation in the GIT, limited uptake of vaccine antigens across the IEC barrier and the generally poor immunogenicity of oral vaccines [26], necessitating the identification and inclusion of effective orally active adjuvants. We demonstrate that protection of antigens alone within SmPill® is insufficient to enhance immunity but our integrated system incorporating a candidate ETEC vaccine antigen and the invariant natural killer (iNK) T cell activator, alpha-galactosylceramide (α-Galcer) combines antigen protection, controlled release and immune-potentiating activity after three rounds of immunization. Advanced characterisation reveals the internal structure of this system with antigens associated with and released in the dispersed phase. This novel system thus addresses the key challenges to oral vaccine delivery in an integrated manner.

2. Material and methods

2.1. Manufacture of SmPill®

SmPill® mini-spheres were manufactured as previously described [32] and summarized in Fig. 1. Briefly, when included in a formulation, α-Galcer (Avanti Polar Lipids Inc., USA) was dissolved in Kolliphor HS 15 (BASF GmbH, Germany) at 40 °C under magnetic stirring. JT-49 (generously provided by Prof. Ann-Marie Svennerholm) or JT-227 in a PBS suspension was then added and the resulting dispersed phase mixed until homogeneous. Sorbitol (Roquette Freres, France) was dissolved in water at room temperature, then type A porcine gelatine (Nitta Gelatin NA Inc., USA) was added, the temperature was increased to 60–70 °C and the mixture stirred until complete dissolution of the components. The aqueous phase and dispersed phase were mixed at 50–55 °C to achieve homogeneity. The homogeneous solution was ejected through a single orifice to form droplets that fell into a cooling oil medium (Miglyol 810N, Cremer Oleo GmbH;Co. KG, Germany) at 8–10 °C. After approximately 30 min, the mini-spheres were recovered from the cooling oil medium, centrifuged to eliminate excess oil and dried at room temperature. Mini-sphere size after drying was between 1 and 2 mm. SmPill® mini-spheres were coated using a Vector MFL.01 Fluid Bed System in the bottom spray configuration with Eudragit

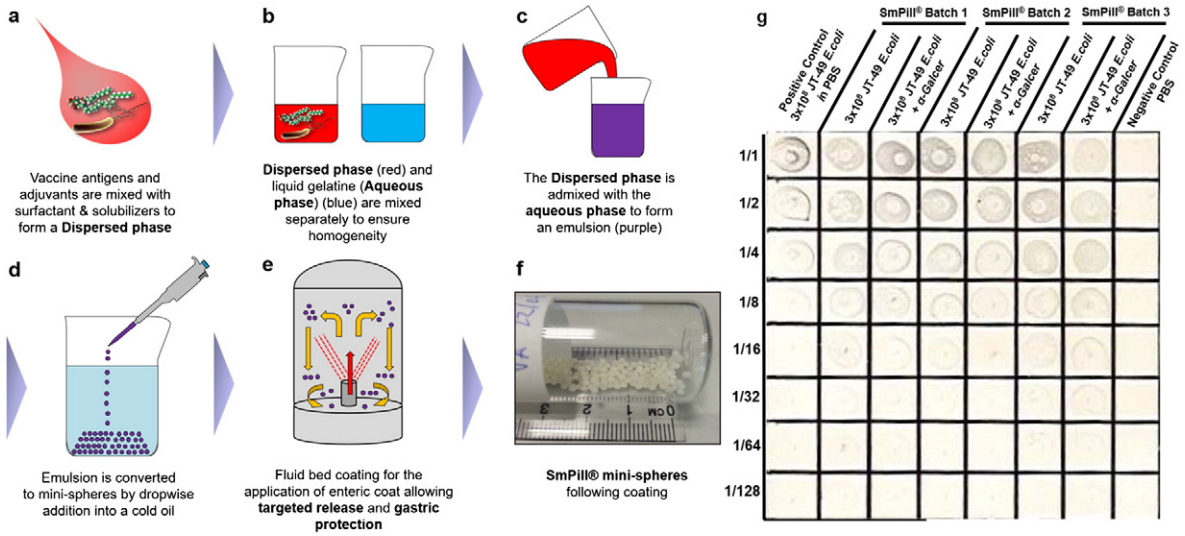

Fig. 1. SmPill® manufacture and super-structure. The Single-multiple Pill (SmPill®) mini-spheres are manufactured using a multi-step process. (**a**) Vaccine components (antigen and adjuvant) are combined within a suitable solubilizing agent (e.g. Kolliphor HS 15) which forms the dispersed phase. (**b**) Gelatine is dissolved in water together with plasticizers to form the aqueous phase. Both phases are (**c**) combined to form an emulsion. (**d**) This emulsion is added drop-wise into a chilled oil composed of medium chain fatty acids. This process yields (**d**) mini-spheres containing the dispersed phase within the now solid aqueous phase. After drying (**e**), beads are coated using a fluid bed system with a bottom spray configuration with a chosen enteric polymer to protect against acidic gastric pH and to allow for controlled release in discrete regions within the intestine. (**f**) Mini-spheres ranging in size from 1-2 mm are the outcome of this process. (**g**) SmPill® mini-spheres containing 3×10^8 whole cell killed JT-49 with or without α-Galcer were dissolved in PBS at 37 °C to release into suspension. Compared to a reference control of 3×10^8 JT-49 in PBS, dot-blots against CFA/I derived from SmPill® supernatants appeared similar showing accurate and reproducible loading of vaccine components is possible across three independent production cycles. Most importantly the CFA/I-mAb used in the dot blots recognized the epitope on JT-49 in SmPill® confirming the intact loading of the components which is often a concern with some oral delivery methods.

L30D 55 (Evonik Industries AG, Germany). The weight gain of polymer achieved was between 5 and 8%. Bacterial content of SmPill® was confirmed by CFA/I-specific dot blot for each batch. Finalized batches of SmPill® were stored dry and at room temperature until use.

2.2. Microscopic analysis of SmPill®

SmPill® were embedded in TissueTek OCT compound (Sakura Finetek) and snap frozen in liquid nitrogen. Sections (7 μm) were blocked in phosphate-buffered saline (PBS)-1% Bovine serum albumin (BSA) for 30 min and stained for 1 h with mouse anti-CFA/I mAb (supplied by Prof. Ann-Marie Svennerholm) diluted 1:25 in PBS-0.1% BSA followed by FITC-conjugated anti-mouse IgG (eBioscience) diluted 1:50 in PBS-0.1% BSA for 1 h. The sections were observed using the Olympus FV1000 scanning confocal (SBI microscopy facility, TBSI). SmPill® were also analysed by electron microscopy after being osmicated in OsO4 and embedded in Epon. Ultra-sections were gold coated for 30 s and directly observed using a Hitachi S-4300 Field Emission Scanning Electron Microscope (Conway Institute, UCD).

2.3. Bacterial release from SmPill®

SmPill® were incubated in simulated intestinal fluid (6.8 g/l NH_2PO_4, 7.7% 0.2 N NaOH, 10 g/l pancreatin; pH 6.8) or simulated gastric fluid (2 g/l NaCl, 3.2 g/l porcine pepsin, 0.7% HCl; pH 1.2) at 37 °C with shaking for 1 h. Bacterial release was analysed at various time-points by CFA/I-specific dot-blot assay using mouse anti-CFA/I mAb (supplied by Prof. Ann-Marie Svennerholm) diluted 1:100 in PBS-Tween -0.1% BSA for 2 h followed by HRP-conjugated anti-mouse IgG (Southern Biotech) diluted 1:2000 in PBS-Tween -0.1% BSA for 2 h. Then flow cytometry analysis was performed at various time-points using a CFA/I-positive GFP-expressing ETEC strain (JT-227) and SmPill® containing this bacterial strain. Samples were acquired on a BD Accuri C6 flow cytometer (BD) running FACs Diva (BD). Analysis of samples was performed using FlowJo™ (Tree Star Inc., USA).

2.4. Intestinal explant model

One SmPill® containing GFP^+ ETEC (JT-227) was introduced into the lumen of a 2-cm intestinal explant, tied at both ends to restrain the mini-sphere and incubated in RPMI 1640 medium at 37 °C, 5% CO_2 for 5 or 30 min. The explant containing the SmPill® was fixed in 4% paraformaldehyde/PBS overnight at 4 °C. After incubation in 12% sucrose/PBS for 3 h and 20% sucrose/PBS overnight at 4 °C, the explant was embedded in TissueTek optimal cutting temperature compound and snap frozen in liquid nitrogen. Sections (7 μm) were observed using an Olympus BX51 upright microscope and a Leica SP8 gated STED (SBI microscopy facility, TBSI).

2.5. Animals

Female BALB/c mice were obtained from Charles River Laboratories, Inc., and were used at 12–16 weeks of age. Animals were maintained according to the regulations of the EU and the Irish Department of Health and all procedures performed were conducted under animal licence number B100/3321 and were approved by the Trinity College Dublin Animal Research Ethics Committee (Ethical Approval Number 091210).

2.6. Immunization strategy

Groups of mice ($n = 5$) were immunized orally on days 0, 1, 14, 15, 28 and 29 as per Supplementary Fig. 1. One hour prior to immunization food was withdrawn with water provided ad libitum. Mice were orally gavaged with 200 μl of 0.3 M sodium bicarbonate buffer (pH 9) to neutralize stomach acid. After 20 min mice were gavaged with 200 μl sterile PBS containing 3×10^8 WCK JT-49 bacteria per mouse with or without α-Galcer (10 μg) (Avanti Lipids) or CT (10 μg) (Sigma Aldrich) or with PBS alone. Alternatively, mice were vaccinated with SmPill® mini-spheres. Food was again withdrawn 1 h prior to vaccination, however, no bicarbonate buffer was administered. SmPill® mini-spheres containing 3×10^8 JT-49 with or without α-Galcer (10 μg)

were delivered by loading the spheres into the silicon tip of a 17G flexible feeding tube (Agntho's, Sweden) and 50 μl of pH 5 buffer was used to aid delivery. Food was returned to all animals 30 min after vaccination.

2.7. Collection of faecal, saliva and serum samples

Faecal pellet supernatants (FPS) were collected as described previously [24]. Briefly mice were placed into individual cages and 5 fresh faecal pellets collected in 500 μl of cold faecal pellet buffer (0.1 mg/ml Soybean trypsin inhibitor (STI) (Sigma Aldrich), 1% BSA, 25 mM ethylenediaminetetraacetic acid (EDTA) (Gibco), 1 mM PEFABloc (Sigma Aldrich), 50% Glycerol in 1× PBS) and kept on ice for 4 h. Samples were then emulsified with a yellow inoculation needle (Greiner Bio-one), centrifuged at 15,400*g* × 5 min at 4 °C and supernatants stored at −80 °C until further use.

To obtain saliva, mice were injected intra-peritoneally with 0.1 mg/ml of Pilocarpine-HCl (TOCRIS Bioscience) in 100 μl Dulbecco's PBS (BioSera) and carefully observed for the onset of salivation. Saliva was collected in tubes for the duration of salivation, kept on ice, and later stored at −20 °C until further use.

Blood was obtained from mice by incision into the tail vein following heating under a heat lamp for 5 min. Samples were left to coagulate overnight at 4 °C and then centrifuged at 9200*g* × 10 min. The serum was removed and stored at −20 °C until further use.

2.8. Intestinal IgA responses

Intestinal tissue IgA responses were measured using the Perfusion-Extraction (PERFEXT) method previously described [33]. Briefly, mice were sacrificed by CO_2 asphyxiation and perfused with 20 ml 0.1% Heparin-sulphate (Sigma Aldrich)-PBS using a 23G needle (BD) and 20 ml syringe (BD) through the heart and the caudal mesenteric arteries. The intestines were removed, the GIT was trimmed of fat and mesentery and the duodenum removed. Intestinal segments from the jejunum and colon were opened longitudinally to remove faeces and washed in ice cold PBS. Samples were placed in 270 μl ice cold sample buffer (0.1 mg/ml STI, 0.05 M EDTA, 1 mM PEFABloc, 0.1% BSA, 0.05% Tween20 in 1× PBS) and stored on ice. 30 μl of 20% saponin from quillaja bark (Sigma Aldrich) was added into each tube and incubated overnight at 4 °C. Supernatants were isolated following centrifugation at 14,000*g* × 10 min at 4 °C and stored at −20 °C until further use.

Washes were performed on isolated intestines with 6 ml wash buffer (0.1 mg/ml STI, 0.05 M EDTA in 1× PBS) for small or 1 ml for large intestinal washes respectively and washes kept on ice. Samples were centrifuged at 1500*g* × 10 min at 4 °C. 2 ml of small and 0.8 ml of large intestinal supernatant was transferred into tubes containing 100 mM phenylmethanesulfonylfluoride (PMSF) (Sigma Aldrich). Samples were centrifuged at 18,000*g* × 10 min at 4 °C before being transferred to tubes containing a further 100 mM PMSF. 50 μl of fetal calf serum (BioSera) and 5 μl of 1% sodium azide solution were finally added per 1 ml of supernatant and stored at −20 °C until analysis.

2.9. Measurement of CFA/I specific antibody responses

CFA/I-specific antibody titres were determined by antigen-specific enzyme-linked immunosorbent assay. 50 μl per well of 1 μg/ml recombinant CFA/I (supplied by Prof. Ann-Marie Svennerholm) was diluted in 1× PBS and added to 96-well micro-titre plates (medium binding) (Greiner BioOne) and incubated overnight at 4 °C. Plates were washed three times in 1× PBS and blocked with 200 μl 0.1% BSA for 60 min at 37 °C. The blocking solution was flicked from the plates and tapped dry. 50 μl of sample were added to and serially diluted across the plate in 0.1% BSA in 1× PBS-T (0.05% Tween) and incubated overnight at 4 °C. Plates were washed again 3 times in wash buffer (1× PBS + 0.05% Tween 20). 50 μl per well of biotin-conjugated anti-mouse IgA (1:1000) (BD), IgG (1:4000) (Sigma Aldrich), IgG1 (1:5000) (BD), IgG2a (1:5000) (BD) or IgG2b (1:5000) (BD) was added to each well and incubated overnight at 4 °C. Plates were washed three times in wash buffer and 50 μl per well of 700 ng/ml HRP-conjugated streptavidin (Sigma Aldrich) added to plates for 30 min at room temperature. Plates were washed four times in wash buffer and one final time in 1× PBS. 1 mg/ml *o*-Phenylenediamine dihydrochloride substrate (Sigma Aldrich) was prepared in 0.1 M phosphate citrate buffer (pH 5) (heated to 37 °C) containing 4 μl H_2O_2 per 10 ml substrate and 100 μl added per well. The plates were left to develop at room temperature in the dark, the reaction was then stopped by the addition of 25 μl/well of 1 M H_2SO_4 and the absorbance at 492 nm read using a Microplate Reader (Thermo Scientific) running Scan-IT software (Thermo Scientific) to acquire data. Antibody concentrations were expressed as endpoint titres calculated by regression of a curve of OD values versus reciprocal serum levels to a cut-off point of 2 standard deviations above control sera.

2.10. Statistics

The data represented in each graph are arithmetic means, calculated from 5 animals per group. The error bars represent the standard errors of the mean. Analysis of variance (ANOVA) was used to determine significant differences between treatments, and the degree of any significance was calculated by Tukey's multiple comparison test in experiments containing 3 or more groups. A single-tail Student's *t*-test was used to calculate any degree of significance in experiments containing two groups. Area under the curve (AUC) was calculated for each mouse at each time point. AUC represent the arithmetic means of 5 mice. Prism5 (GraphPad) was used for all statistical analysis. *P* values <0.05 were regarded as significant.

3. Results and discussion

3.1. Manufacture and loading of vaccine components into the SmPill®

The manufacturing process of SmPill® vaccine mini-sphere is described in Fig. 1. Antigens and adjuvants were solubilized/suspended to form the dispersed phase (Fig. 1a). Here, Kolliphor HS 15 was used as it has been extensively characterized and its safety has been demonstrated in humans in addition to being commercially available as an excipient [34]. The dispersed phase is combined with an aqueous gelatine phase (Fig. 1c) to form an emulsion that is added drop-wise into a cooling medium (a generic cold oil composed of medium chain fatty acids), resulting in the formation of mini-spheres, thus trapping the dispersed phase micro-droplets in the aqueous gelatine phase which forms the matrix (Fig. 1d). Following a drying process, the mini-spheres receive a pH-resistant enteric coating (Fig. 1e). The ability to select from a variety of different and commercially available enteric coatings allows for the controlled release of the micro-droplets containing the antigen/adjuvant combination. Here EUDRAGIT® L 30 D-55 was selected as an enteric polymer as it was shown to protect a prototype oral cholera vaccine from low pH while facilitating controlled antigen release [35]. The final product of the manufacturing process yields the coated mini-spheres, approximately 1 mm in diameter (Fig. 1f). Dot blot analysis of 3 individual batches of dissolved SmPill® vaccine mini-spheres using an anti-CFA/I monoclonal antibody (mAb) revealed that it is possible to reproducibly incorporate JT-49 into SmPill® mini-sphere across multiple independent production runs (Fig. 1g).

3.2. SmPill® vaccine structural characterization

We have demonstrated the ability of SmPill® to enhance oral delivery of small molecule drugs [30,31] but given the complexity of our vaccine formulation containing a killed bacterial antigen and α-Galcer adjuvant it was essential to perform advanced characterisation

of the SmPill® vaccine formulation to identify and localise the components. The SmPill® comprises a porous sphere, containing cavities thought to contain the payload and surrounded by an exterior enteric coating (Fig. 2a).

Microscopic analysis of SmPill® sections revealed a core structure comprising a matrix of cavities that are heterogeneous in size (Fig. 2b). Scanning electron microscope images of bead sections revealed that these cavities contain micro-droplets of the dispersed phase with aggregates of rod-shaped bacteria within them (Fig. 2c). To confirm the presence of JT-49 in the droplets, SmPill® sections were stained with a fluorescein isothiocyanate (FITC) labelled anti-CFA/I mAb and visualized by confocal microscopy (Fig. 2d). Rod shaped aggregates correlated to green fluorescence when phase contrast and FITC images were overlaid confirming the presence of JT-49 in the micro-droplets (Fig. 2d). Together this demonstrates the loading of antigen into the micro-droplets within the matrix but most importantly that the manufacturing process facilitated the loading of JT-49 into the mini-spheres intact and without compromising the antigenicity of the CFA/I epitope.

3.3. The SmPill® vaccine facilitates controlled antigen release at intestinal pH

Systems facilitating a controlled and sustained release of antigen may better mimic the natural course of infection and could enhance immunogenicity through the generation of a vaccine reservoir allowing for longer antigen exposure to the immune system [36]. SmPill® mini-spheres retain their structural integrity when dry (Supplementary Fig. 2) but once rehydrated, the structure of the gelatine matrix swells and disintegrates, releasing the dispersed phase into the surrounding medium as micro-droplets (Supplementary Fig. 2). Bacterial release from SmPill® was assessed in simulated intestinal fluid (SIF) over a time course of one hour. Initial bacterial release into SIF occurred after 10 min when analysed by CFA/I-specific dot blot (Fig. 3a). Densitometry analysis revealed that following progressive release of JT-49 over a 1 h time course, a peak release of JT-49 occurred after 50 min in SIF from SmPill® (Fig. 3a). In order to better quantify and investigate the release of bacteria in both SIF and simulated gastric fluid (SGF), we utilized a CFA/I-positive ETEC strain expressing green fluorescent protein (GFP)

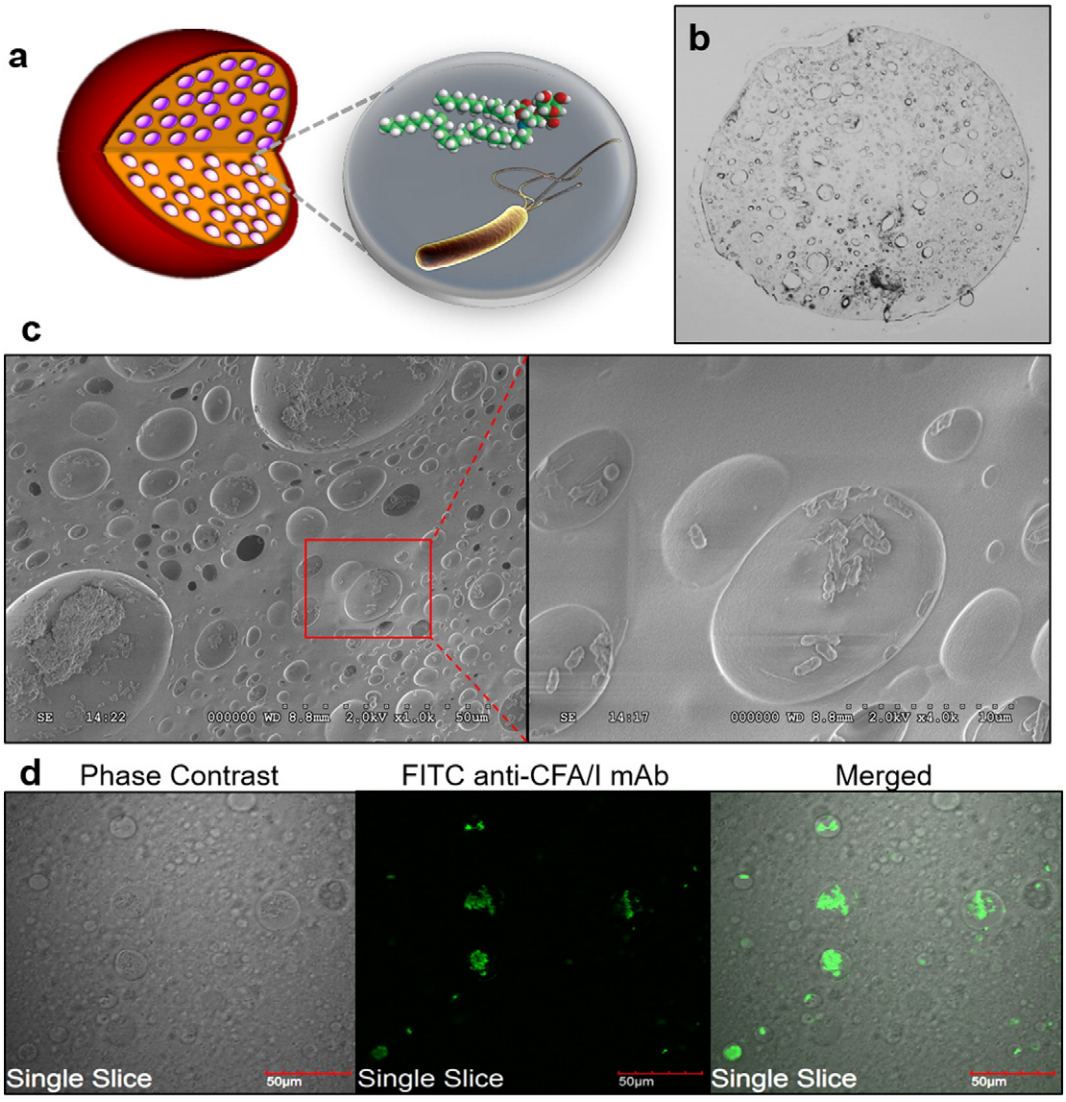

Fig. 2. SmPill® structure and antigen localization. SmPill® is a unique oral delivery system (**a**) composed of an exterior enteric coating (red) which resists degradation at low pH. The gelatine core (yellow) is composed of a matrix of micro-droplets of a dispersed phase (white), which contains the payload. (**b**) The internal structure of SmPill® mini-spheres contain a non-uniform dispersal of micro-droplets varying from 50 μm to 1 μm in size. (**c**) Scanning electron microscopy reveals aggregates of rod-shaped structures contained within the larger micro-droplets. (**d**) Staining of SmPill® sections containing 3×10^8 JT-49 with an anti-CFA/I-FITC mAb and visualization by confocal microscopy confirms the rod-shaped aggregates contained within micro-droplets to be CFA/I-positive JT-49.

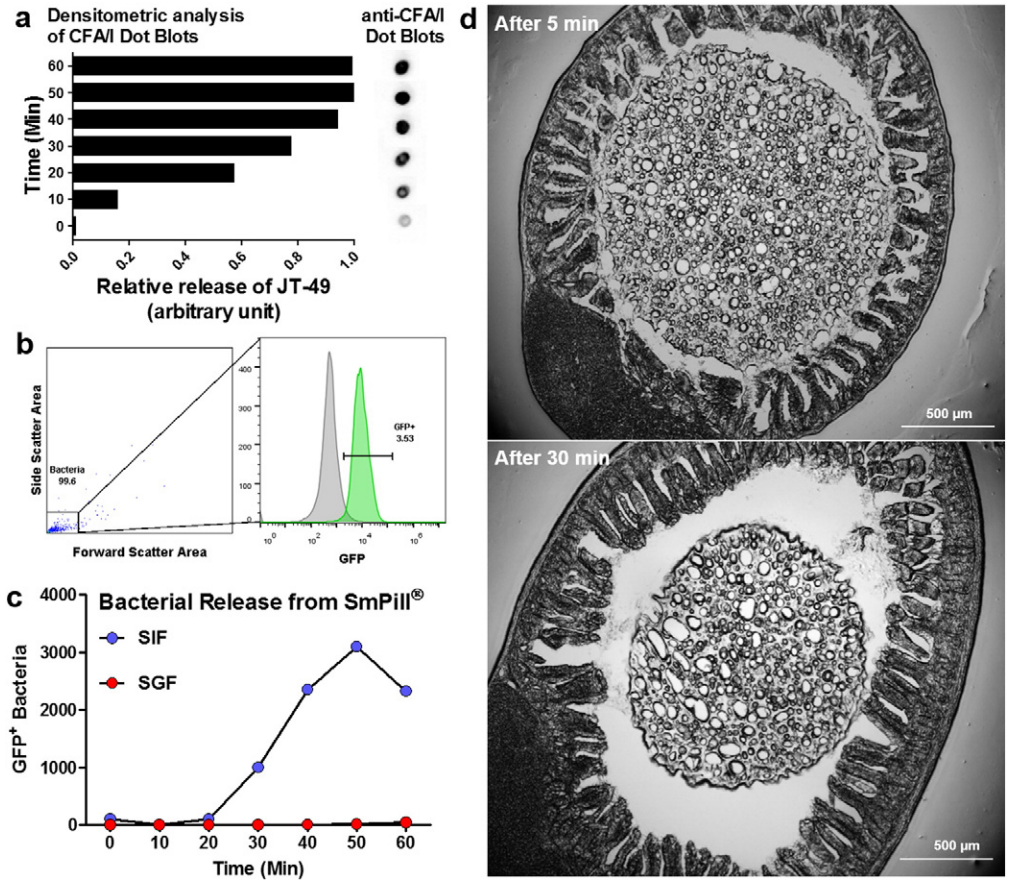

Fig. 3. SmPill®acts as a pH sensitive controlled release system for vaccine formulations in the GIT. JT-49 release (**a**) from SmPill® mini-spheres occurs in a time dependent manner at simulated intestinal pH as evaluated by CFA/I-specific dot blots and quantified by densitometry. (**b**) JT-49 (gray) can be differentiated from the CFA/I-positive GFP-expressing ETEC strain JT-227 (green) by flow cytometry. (**c**) No GFP^+ positive JT-227 release is detected under simulated gastric conditions however controlled release of GFP^+ bacteria is detected in simulated intestinal fluid (SIF) peaking at 50 min. (**d**) After 5 min in an intestinal explant model, the SmPill® structure remains largely intact with dispersed phase pockets at the intestinal interface appearing to have released their contents. Following a 30 min incubation in the explant, the size of SmPill® mini-sphere has reduced 1.4 fold while the matrix structure of the mini-sphere is retained.

(JT-227). Flow cytometric analysis of inactivated JT-49 and JT-227 revealed that after formalin treatment, GFP fluorescence can be detected in JT-227 but not JT-49 (Fig. 3b). Moreover, when quantified by flow cytometry, JT-227 followed a similar release profile from SmPill® in SIF as JT-49, peaking at 50 min (Fig. 3c). In contrast, no bacterial release was observed in SGF over the course of 1 h, indicating that SmPill® mini-spheres retain their structural integrity in an acidic pH for an extended period of time (Fig. 3c). In the presence of mechanical agitation to simulate the gastric contractions in the stomach and peristalsis in the duodenum, SmPill® retained its structural integrity, thus protecting its contents from early release due to physical degradation of the delivery system (Fig. 3c).

In order to better understand the release mechanism of SmPill® and its interaction with the intestinal environment, we developed an *ex vivo* intestinal explant model. 2 cm sections of freshly isolated small intestine were tied off at one end and one SmPill® inserted into the lumen. To retain the SmPill® in the lumen the other end was also tied off. Explants were placed into RPMI medium and incubated at 37 °C with shaking to simulate peristalsis. After incubation, explants were embedded and sectioned to visualize the SmPill® structure after interaction with the intestinal environment. After 30 min in this intestinal explant model (Fig. 3d), the physical size of SmPill® was reduced compared to that seen after 5 min (Fig. 3d). Interestingly, close contact of SmPill® mini-spheres with villi was observed after 30 min (Figs. 3d; 4a), likely caused by the swelling and dissolution of the gelatine core in the hydrated and warm environment of the intestine. Whether this mechanism contributes directly to antigen uptake however remains to be elucidated. These results, taken together with the gradual release kinetics (Fig. 3) demonstrate that the SmPill® platform possesses an intrinsic delayed release mechanism that is selective for the higher pH of the distal small intestine. In addition to providing safe passage through the harsh gastric environment and controlled release in the small intestine, SmPill® micro-spheres may act as a luminal reservoir allowing for persistent vaccine release over an extended period of time. The formation of a vaccine reservoir has been suggested to enhance efficacy via the persistence of vaccine material at the site of antigen uptake [36]. This depot-like effect leads to longer combined antigen/adjuvant exposure over time and a greater chance that both components will encounter cells of the innate immune system in a synchronized manner. Considering that the structure of the beads remains intact even after 30 min (Fig. 3d), it is plausible that the SmPill® may impart such a reservoir function on its contents.

3.4. Ex vivo release studies

Antigen release from the SmPill® occurs via the dissolution of the gelatine matrix and the release of micro-droplets of the dispersed phase containing the antigen and adjuvant mixture into the surrounding environment (Supplementary Fig. 2). Intimate contact between the gelatine phase and the intestinal epithelial surface is observed at the villous-SmPill® interface (Fig. 4a). Interestingly, this intimate contact also correlates with the presence of GFP-fluorescence at the tips of the villi that are in contact with SmPill® micro-spheres containing JT-227 (Fig. 4a). While penetration through the mucus layers of the GIT

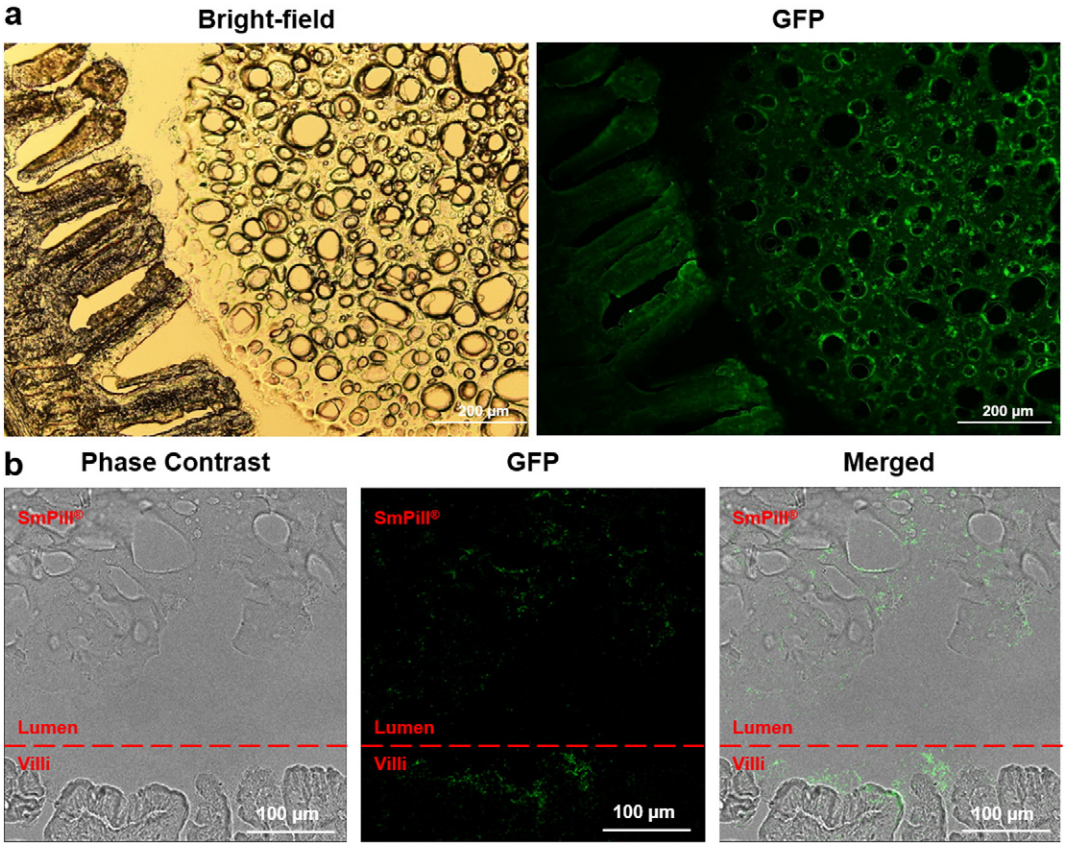

Fig. 4. Interaction of SmPill® derived oil droplets with the villous surface. (**a**) GFP$^+$ JT-227 released from SmPill® in intestinal explants can be observed at the tips of the villi at intestinal-SmPill® interface. Translocation (**b**) of JT-227 can be observed from micro-droplets in the SmPill®, across the intestinal lumen into the villi of the epithelium as visualized by confocal microscopy.

has been shown to be a considerable challenge when delivering oral vaccines [26], the potential of SmPill® to permit close contact with the intestinal epithelium may enhance the uptake of the antigens and adjuvant. We imaged this interface and found that the distance between the SmPill® micro-spheres and intestinal epithelium is at some points less than 100 μm (Fig. 4b). Considering that the relative thickness of the mucus layer varies from 10 μm in the ileum to 100 μm in the colon, the SmPill® platform could help to overcome this barrier. Moreover, JT-227 bacteria were found inside the villi, suggesting enhanced antigen uptake following release from SmPill® (Fig. 4b). The potential to enhance antigen/adjuvant delivery directly at the mucosal epithelium may limit dilution of vaccine components by gastrointestinal secretions and mucus, which may render the dose of vaccine arriving intact at sites of immune surveillance suboptimal for stimulating immune responses [26]. Formulation of the antigen and adjuvant in SmPill® resulted in the concentrated delivery of JT-227 to the intestinal epithelium, indicating that the delivery system may provide additional protection of contents from dilution.

3.5. Identification of α-Galcer as a potent oral adjuvant

Adjuvants are utilized in vaccine formulations in order to enhance the immunogenicity of non-living antigens [16,37]. However, while a number of adjuvants are licensed for use in injectable vaccines, there are currently no adjuvants included in clinically applied oral vaccines [37]. Due to their remarkably potent ability to drive immune responses to orally administered antigens, cholera toxin (CT) (and LT) are regarded as the "gold standard" experimental mucosal adjuvants [38]. However, in their unmodified native forms their inherent toxicity precludes the clinical use of CT and LT [16]. As a result, there is an urgent need to identify safe and potent adjuvants, particularly for delivery to the gastrointestinal tract. While a detoxified double mutant variant of LT has shown promise in early clinical trials following oral administration [39], there are additional challenges associated with both the delivery of labile components and their inclusion into delivery systems [26]. These challenges demand novel delivery approaches.

Recently populations of unconventional T cells have received much interest as possible adjuvant targets for mucosal vaccination due to the availability of well characterized activating ligands, their ability to drive potent immune responses following activation and their anatomical positioning at the mucosal interface of the gastrointestinal tract (GIT) [40]. Both iNKT cells and mucosal associated invariant T cells have potential as target cells to enhance immune responses to orally delivered antigens [40,41]. To this end, we investigated the oral adjuvanticity of the well characterized iNKT cell activating glycolipid [41,42], α-Galcer and compared this to CT following three rounds of oral vaccination together with JT-49 as outlined in Supplementary Fig. 1. Thirty four days after initial vaccination, enhanced faecal anti-CFA/I IgA titres (a direct measure of intestinal antibody responses [43]) were induced by JT-49 admixed with either α-Galcer or CT compared to administration of the bacteria alone (Supplementary Fig. 3). In these studies α-Galcer was as effective an adjuvant as CT in promoting intestinal IgA responses (Supplementary Fig. 3).

3.6. Enhancement of intestinal responses following oral vaccination

Having extensively characterized SmPill® mini-spheres and established their capacity to protect antigens from low pH and facilitate controlled release, we sought to determine if encapsulation of JT-49 and α-Galcer could enhance immunogenicity following oral vaccination.

CFA/I-specific IgA titres were not only significantly enhanced following encapsulation in SmPill® micro-spheres versus delivery in solution (Fig. 5a), but the rate at which these titres were established over time, as determined by measuring the area under the curve (AUC), was significantly more rapid following vaccination with JT-49 and α-Galcer in SmPill® (Fig. 5b). Considering that ETEC outbreaks can occur in the aftermath of natural disasters or conflict, developing a vaccine capable of delivering a rapidly induced and potent immune response is highly desirable. Delivery of an OEV formulation by SmPill® not only enhances the immunogenicity but also enhances the speed at which an immune response is elicited. Rapid establishment of herd immunity during a natural disaster or emergency can prevent outbreaks of enteric disease which can add additional hardship to a population already suffering in the aftermath of such events. The ability to accurately dose and easily administer SmPill® combined with the rapid establishment of immunity make it an attractive OEV delivery system for use in resource poor settings.

It has been claimed that some oral delivery systems have an intrinsic adjuvant capacity in the absence of an exogenous immuno-potentiator [28,44]. While shielding a formulation from degradation is a key objective when developing efficacious oral vaccines, it is unclear whether this alone is sufficient to generate effective oral vaccine systems. When α-Galcer was removed from the SmPill® formulation, a significant reduction in antigen-specific faecal IgA was observed (Fig. 5c; d). This suggests that delivery of an antigen by SmPill® in isolation is not sufficient to elicit strong intestinal immune responses. However, with the addition of solubilized α-Galcer, a potent enhancement of the formulations ability to drive gut responses is observed (Fig. 5c; d). Although a SmPill® formulated drug showed enhanced efficacy against colitis in a mouse model [30,31], these results clearly demonstrate that in the context of oral vaccination, targeted delivery alone is not sufficient to elicit a potent immune response.

We next sought to measure the induction of antibody responses against CFA/I in the jejunum in order to ascertain if immune responses were induced at the site of ETEC colonization. The Perfusion-Extraction (PERFEXT) technique allows for the quantification of a local antibody and cytokine response in a tissue of interest *in vivo* [33]. This technique is also amenable to quantifying antigen-specific IgA in intestinal tissue following oral vaccination [24]. Following perfusion with heparin-phosphate buffered saline (PBS) (to exclude serum IgA contamination), jejunal tissue sections were treated with saponin to permeablize the tissue and release IgA into the supernatant. A strong induction of anti-CFA/I IgA was detected in jejunal tissue supernatants following oral vaccination with JT-49 and α-Galcer in SmPill® (Fig. 5e). Furthermore, we also detected high CFA/I-specific IgA titres in small intestinal washes (Fig. 5f). These results indicated that JT-49 and α-Galcer in SmPill® induced a strong IgA response in the intestinal tissue (Fig. 5e), and effective IgA secretion into the jejunal lumen (Fig. 5f), where it would encounter ETEC in the context of a bacterial infection.

3.7. Induction of mucosal immune responses at distant effector sites

While ETEC establishes infection in the jejunum, other pathogenic strains of *E. coli* (such as enterohaemorrhagic and enteropathogenic *E. coli*) colonize more distal sites such as the ileum and the colon respectively [12]. Furthermore, a powerful immune response triggered at an inductive site can potentially elicit strong immune responses at a distant effector site in the GIT. We sought to determine the extent of dissemination of the mucosal immune response elicited following oral immunization with the SmPill® vaccine formulation as a proof of concept that this system may be amenable to vaccination against other enteric infections in the GIT. Following vaccination we detected strong CFA/I-specific antibody responses in both colonic tissue after perfusion (Fig. 6a) and in colonic washes (Fig. 6b). CT, LT and their derivatives can induce immune responses not only along the length of the GIT, but also at distant mucosal sites such as in vaginal secretions [45] supporting the concept of a "common mucosal immune system" [46]. To this end we investigated the ability of SmPill®-based OEV to drive enhanced salivary IgA responses. The vaccine elicited an antigen-specific IgA response in the saliva of mice following oral vaccination (Fig. 6c). Importantly, salivary IgA has been demonstrated to be a non-invasive correlate for the successful induction of intestinal immune responses following infection with wild type ETEC in human volunteers [47]. Additionally, salivary IgA responses have been used as a correlate of intestinal immunity following oral vaccination against enteric pathogens in human trials [48].

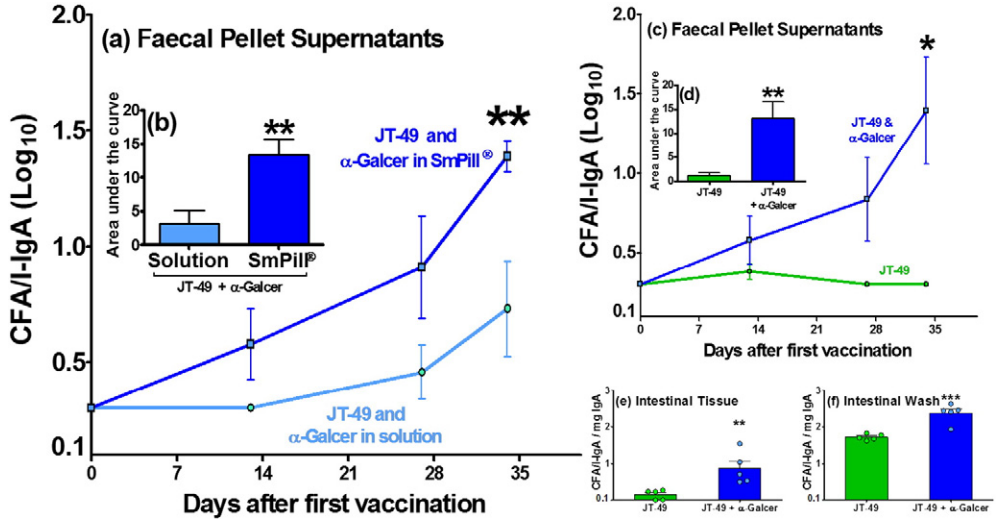

Fig. 5. Enhancement of oral vaccine-mediated mucosal antibody responses following encapsulation of formulations in SmPill®. Mice ($n = 5$) were vaccinated orally with SmPill® mini-spheres or bicarbonate solution containing 3×10^8 whole cell killed JT-49 and 10 μg α-Galcer. (**a**) Faecal pellet supernatants were analysed for CFA/I-specific IgA antibody titres by ELISA. Mice receiving the vaccine by SmPill® had significantly higher CFA/I-specific titres than mice vaccinated with solution. (**b**) Furthermore more rapid induction of this IgA response, determined by area under the curve (AUC) analysis, was detected in SmPill® vaccinated mice compared to mice receiving the formulation in solution. (**c**) Higher CFA/I-specific IgA titres and (**d**) a more rapid induction of faecal IgA responses were dependent on the inclusion of solubilized α-Galcer in SmPill®. Strong local intestinal anti-CFA/I titres were also detected in (**e**) jejunal extracts of mice vaccinated with JT-49 and solubilized α-Galcer in SmPill® following perfusion to exclude serum IgA contamination and in (**f**) intestinal washes from the same mice. Figures are representative of three independent *in vivo* studies.

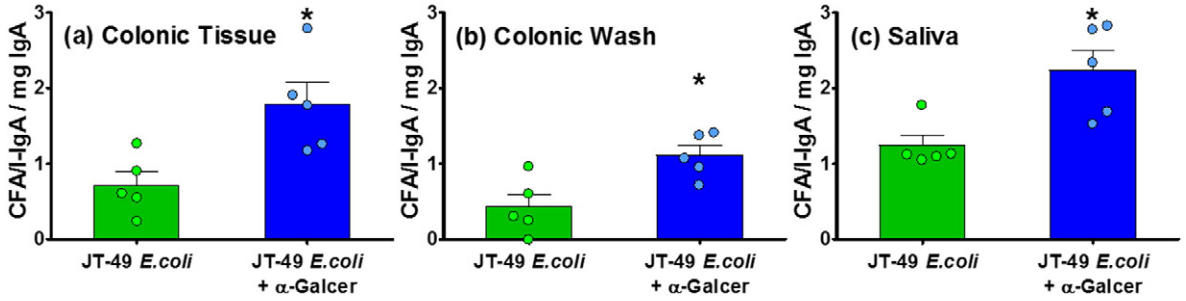

Fig. 6. Large intestinal antibody responses are enhanced by delivery of antigen in α-Galcer adjuvanted SmPill®. Following three rounds of oral vaccination ($n = 5$), SmPill® containing 3×10^8 JT-49 and solubilized α-Galcer (10 μg), mice ($n = 5$) elicited stronger anti-CFA/I titres in (**a**) colonic extracts following perfusion to exclude serum IgA contamination and in colonic washes (**b**) than the formulation without α-Galcer. (**c**) Enhanced salivary CFA/I-specific IgA responses were also detected in mice immunized with antigen in α-Galcer-adjuvanted SmPill® but not when antigen was administered in capsules without adjuvant. Figures are representative of three independent *in vivo* studies.

3.8. Evaluation of serum antibody responses

Following oral vaccination with a candidate ETEC vaccine, studies have found that antigen-specific IgA-producing plasma cells can be detected in the blood and suggested that these cells are most likely of mucosal origin [49–51]. We observed a rapid induction of CFA/I-specific serum IgA following oral vaccination with JT-49 and α-Galcer in SmPill®, which was dependent on the presence of α-Galcer in the formulation (Fig. 7a; b). While ETEC is non-invasive, other enteric pathogens invade through the intestinal mucosa and manifest themselves as a systemic infection. Mucosal vaccination has the potential to elicit immune responses in both the mucosal and systemic immune compartments [16,37]. Here, high titres of antigen-specific IgG were detected following oral vaccination with JT-49 and α-Galcer in SmPill® (Fig. 7c), a profile dominated by IgG1 (Fig. 7d–f).

3.9. A proposed mechanism for the efficacy of an integrated approach to oral vaccination

While the challenges associated with vaccine delivery by the oral route have to date been largely addressed in isolation, the current approach focussed on overcoming these in an integrated manner whereby a SmPill® based vaccine protects the antigen through the low pH of the stomach (Fig. 8a). Following passage of the mini-spheres through the stomach and arrival in the more neutral pH environment of distal parts of the small intestine, the enteric coating begins to degrade (Fig. 8b), exposing the gelatine core of the SmPill® and leading to droplet release (Fig. 8c). Uniquely combined within these droplets are the CFA/I-overexpressing formalin killed JT-49 and the solubilized adjuvant, α-Galcer. Co-delivery of these components across the IEC barrier leads to the generation of potent adaptive immune responses characterized by strong IgA induction in the intestinal tissue (Fig. 8d–g). Intestinal ETEC infection (Fig. 8h–i) would then trigger the delivery of CFA/I-specific SIgA into the intestinal lumen (Fig. 8j). Here in the context of natural infection, CFA1-specific SIgA can bind to and neutralize the invading ETEC bacteria (Fig. 8k).

4. Conclusions

Incidences of enteric diarrhoea are predicted to rise globally. This has been attributed in part to global warming, which is creating new environmental niches for bacteria such as *V. cholerae* and ETEC allowing them to thrive in regions where these infections are usually not encountered [52]. Additionally, large scale human migration and displacement due to famine and conflict is leading to lapses in vaccination schedules and the spreading of infectious diseases. More worrying is that this migration could lead to the introduction of new pathogens into territories not equipped to intervene with such infections. These challenges demand innovative public health measures to control, contain and

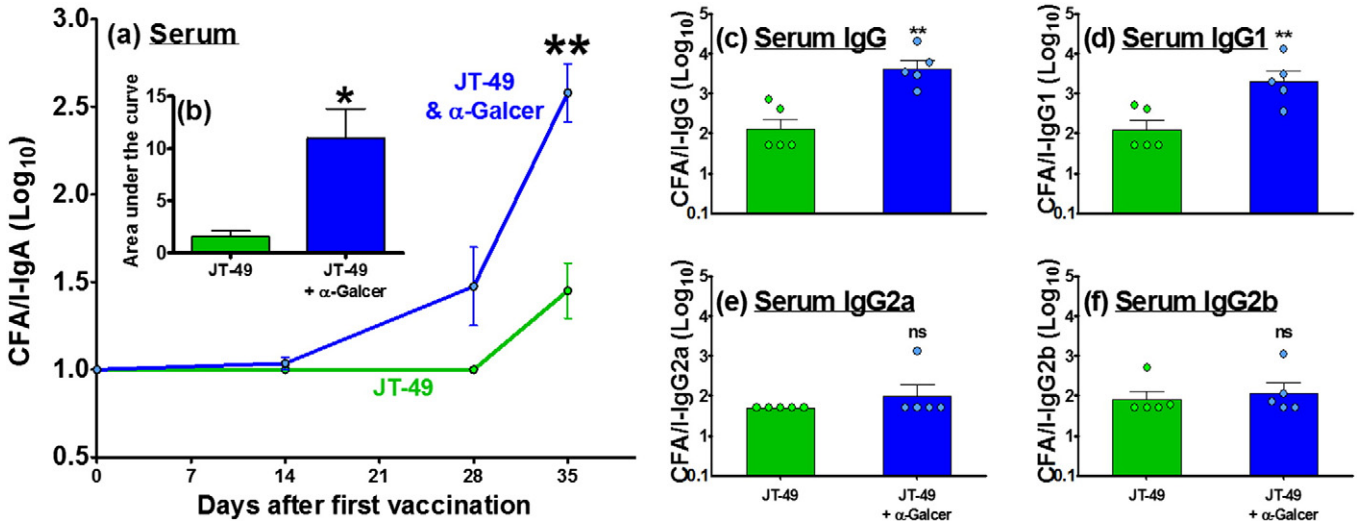

Fig. 7. Enhanced serum IgA responses following oral vaccination with SmPill®requires the inclusion of solubilized α-Galcer as an adjuvant. When mice (n = 5) were vaccinated with SmPill® containing 3×10^8 JT-49 and solubilized α-Galcer (10 μg), anti-CFA/I IgA was detected in the (**a**) serum. Additionally this response (**b**) was elicited rapidly as determined by area und the curve analysis. However, this enhanced response was also dependent on the inclusion of α-Galcer in the formulation. Potent serum (**c**) IgG responses were also detected in mice receiving SmPill® containing JT-49 and α-Galcer. When the subtype was characterized (**d**–**f**) it was determined to be biased towards IgG1, which was dependent on the inclusion of α-Galcer in SmPill®. Figures are representative of three independent *in vivo* studies.

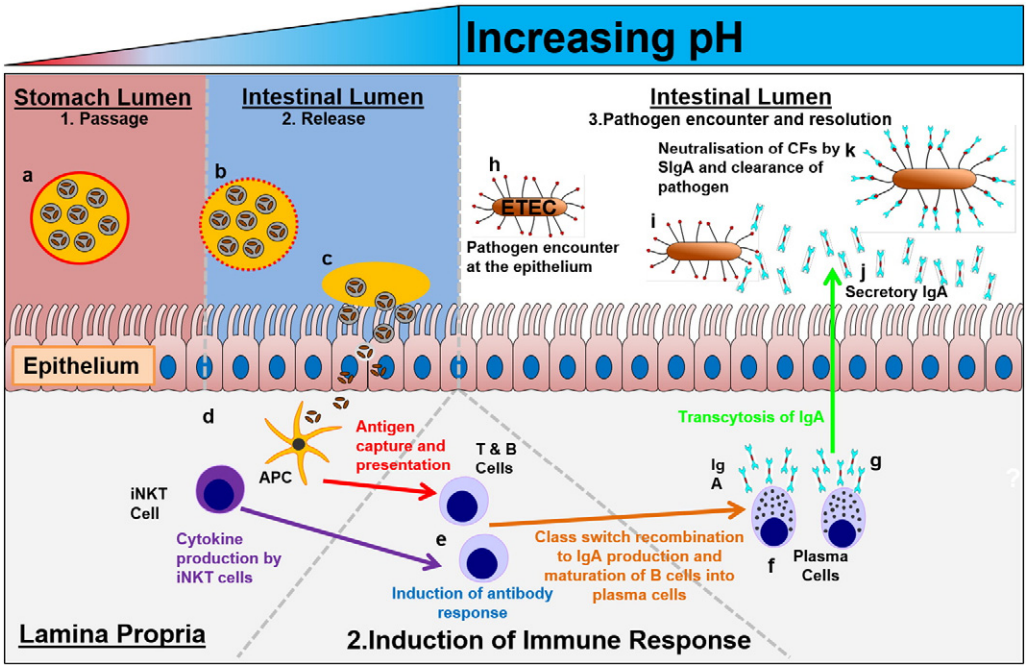

Fig. 8. Proposed mechanism of SmPill® activity and induction of intestinal immune responses. (**a**) The enteric coating on SmPill® mini-spheres allows them to remain stable and intact in the acidic pH of the stomach. (**b**) On exiting the stomach and passage into the increasing pH of the upper portions of the small intestine, the enteric coating begins to degrade exposing the gelatine core. (**c**) The gelatine core begins to be degraded in the small intestine environment releasing the micro-droplets containing the vaccine antigen/solubilized adjuvant in the dispersed phase. (**d**) The micro-droplets are taken up allowing for the passage for antigens and adjuvants across intestinal epithelial cells (IECs) where presentation of the former to antigen-presenting cells (APCs) and the latter to invariant natural killer cells (iNTKs) occurs. (**e**) Together the antigen-loaded APCs and activated iNTKs induce antigen-specific T and B cell responses. (**f**) B cells undergo affinity maturation, class switch recombination and differentiation into plasma cells, which enter into the circulation and eventually home back to and seed in the lamina propria where (**g**) IgA secretion occurs. (**h; i**) Upon infection with viable enterotoxogenic *E. coli* (ETEC) (**j**) antigen-specific secretory IgA (SIgA) is transported into the intestinal lumen where it can (**k**) bind to and neutralize colonization factor antigens (CFAs) expressed by ETEC.

eliminate these pathogens before they can pose a severe health risk. Next generation vaccines, amenable to large scale public administration would be an invaluable tool in the fight against such threats. Despite improvements in water and sanitation facilities and other interventions against enteric disease in endemic regions, global diarrhoea mortality rates have changed little in two decades [53]. Importantly there is a push by the WHO to reduce the use of antibiotics in the treatment of ETEC due to the appearance of antibiotic resistance. The detection of antibiotic resistant ETEC strains at waste water treatment plant effluents suggests that these could become a reservoir of resistant ETEC strains, driving a need for new treatments and prophylactics [54].

While an integral part of experimental vaccine evaluation is proving their protective efficacy in infection models, a reliable mouse protocol for experimental ETEC infection is lacking or less than ideal [55]. Despite this, promising OEV candidates, which display a potent induction of mucosal immune responses in mice have progressed to clinical trials without the need for animal challenge studies. This was further reiterated by a recent conference report recommending a rapid move to human testing of effective ETEC vaccine candidates [2]. The WCK bacterial strains under evaluation in clinical trials overexpress key ETEC CFAs [56] and have been shown to successfully enhance mucosal immune responses when adjuvanted with a double mutant of LT [39]. Although in this study we only evaluate our delivery system with the CFA/I expressing JT-49 strain, we believe a viable candidate vaccine will include other CFs in addition to CFA/I such as CS3, CS5 and CS6, which would cover 90% of all ETEC strains together with an LTB toxoid for anti-toxic immunity. The current study demonstrates the potential of an integrated approach to oral vaccination using the SmPill® to enhance the effectiveness of such a vaccine in a format amenable to convenient and large scale administration to humans. The oral delivery system protects the vaccine antigen and adjuvant components as they pass through the harsh environment of the upper regions of the GIT and controls the release of antigen and solubilized adjuvant together in the small intestine. α-Galcer was comparable to the "gold standard" mucosal adjuvant CT at enhancing CFA/I-specific intestinal immune responses against a novel ETEC antigen, without the toxicity associated with CT. Our approach has demonstrated the need to view the challenges associated with vaccine delivery via the oral route in an integrated manner. A greater understanding of the immune mechanisms directing protective mucosal immunity in the GIT in addition to the application of the advanced SmPill® vaccine approach offers hope that the development of improved non-living vaccines against enteric infections is an attainable goal.

Competing financial interests

ISC is CEO of Sigmoid Pharma Limited, a drug delivery company which holds the rights for the SmPill® integrated drug delivery system used in this manuscript. All other authors have declared no conflicts of interest.

Acknowledgements

We would like to acknowledge the generosity of Prof. Ann-Marie Svennerholm for providing us with the strain JT-49 and the anti-CFA/I mAb. This work was supported by the Irish Research Council (Enterprise partnership scheme postgraduate award (IRCSET-Sigmoid 1010-01)), Science Foundation Ireland (SFI) under Grant number 12/IA/1421 and the SFI Research Centre, Advanced Materials and BioEngineering Research (AMBER) under Grant number SFI/12/RC/2278, and the European Union FP7 program HELICOVAXOR (2013), FP7-SME-2012-1 (Research for SMEs).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2016.05.001.

References

[1] A.C. Cheng, J.R. McDonald, N.M. Thielman, Infectious diarrhea in developed and developing countries, J. Clin. Gastroenterol. 39 (2005) 757–773.
[2] R.I. Walker, A. Clifford, Recommendations regarding the development of combined enterotoxigenic *Escherichia coli* and *Shigella* vaccines for infants, Vaccine 33 (2015) 946–953.
[3] W.A. Petri Jr., M. Miller, H.J. Binder, M.M. Levine, R. Dillingham, R.L. Guerrant, Enteric infections, diarrhea, and their impact on function and development, J. Clin. Invest. 118 (2008) 1277–1290.
[4] C.F. Lanata, C.L. Fischer-Walker, A.C. Olascoaga, C.X. Torres, M.J. Aryee, R.E. Black, O. Child Health Epidemiology Reference Group of the World Health, Unicef, Global causes of diarrheal disease mortality in childrent 5 years of age: a systematic review, PLoS One 8 (2013) e72788.
[5] W.C. Huang, B.M. Hsu, P.M. Kao, C.W. Tao, Y.N. Ho, C.W. Kuo, Y.L. Huang, Seasonal distribution and prevalence of diarrheagenic *Escherichia coli* in different aquatic environments in Taiwan, Ecotoxicol. Environ. Saf. 124 (2016) 37–41.
[6] K.L. Kotloff, J.P. Nataro, W.C. Blackwelder, D. Nasrin, T.H. Farag, S. Panchalingam, Y. Wu, S.O. Sow, D. Sur, R.F. Breiman, A.S. Faruque, A.K. Zaidi, D. Saha, P.L. Alonso, B. Tamboura, D. Sanogo, U. Onwuchekwa, B. Manna, T. Ramamurthy, S. Kanungo, J.B. Ochieng, R. Omore, J.O. Oundo, A. Hossain, S.K. Das, S. Ahmed, S. Qureshi, F. Quadri, R.A. Adegbola, M. Antonio, M.J. Hossain, A. Akinsola, I. Mandomando, T. Nhampossa, S. Acacio, K. Biswas, C.E. O'Reilly, E.D. Mintz, L.Y. Berkeley, K. Muhsen, H. Sommerfelt, R.M. Robins-Browne, M.M. Levine, Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case–control study, Lancet 382 (2013) 209–222.
[7] D.A. Sack, D.C. Kaminsky, R.B. Sack, I.A. Wamola, F. Orskov, I. Orskov, R.C. Slack, R.R. Arthur, A.Z. Kapikian, Enterotoxigenic *Escherichia coli* diarrhea of travelers: a prospective study of American Peace Corps volunteers, Johns Hopkins Med. J. 141 (1977) 63–70.
[8] C.K. Porter, D. Choi, B. Cash, M. Pimentel, J. Murray, L. May, M.S. Riddle, Pathogen-specific risk of chronic gastrointestinal disorders following bacterial causes of foodborne illness, BMC Gastroenterol. 13 (2013) 46.
[9] C.K. Porter, N. Thura, M.S. Riddle, Quantifying the incidence and burden of postinfectious enteric sequelae, Mil. Med. 178 (2013) 452–469.
[10] PATH, The Case for Investment in Enterotoxigenic *Escherichia coli* Vaccines, PATH, Seattle WA, 2011.
[11] W. Gaastra, A.M. Svennerholm, Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC), Trends Microbiol. 4 (1996) 444–452.
[12] M.A. Croxen, B.B. Finlay, Molecular mechanisms of *Escherichia coli* pathogenicity, Nat. Rev. Microbiol. 8 (2010) 26–38.
[13] A.M. Svennerholm, Y.L. Vidal, J. Holmgren, M.M. McConnell, B. Rowe, Role of PCF8775 antigen and its coli surface subcomponents for colonization, disease, and protective immunogenicity of enterotoxigenic *Escherichia coli* in rabbits, Infect. Immun. 56 (1988) 523–528.
[14] F. Qadri, A. Saha, T. Ahmed, A. Al Tarique, Y.A. Begum, A.M. Svennerholm, Disease burden due to enterotoxigenic *Escherichia coli* in the first 2 years of life in an urban community in Bangladesh, Infect. Immun. 75 (2007) 3961–3968.
[15] W.H. Mosley, K.M. Aziz, A.S. Mizanur Rahman, A.K. Alauddin Chowdhury, A. Ahmed, M. Fahimuddin, Report of the 1966–67 cholera vaccine trial in rural East Pakistan, Bull. World Health Organ. 47 (1972) 229–238.
[16] M.R. Neutra, P.A. Kozlowski, Mucosal vaccines: the promise and the challenge, Nat. Rev. Immunol. 6 (2006) 148–158.
[17] R.E. Black, M.M. Levine, M.L. Clements, C.R. Young, A.M. Svennerholm, J. Holmgren, Protective efficacy in humans of killed whole-vibrio oral cholera vaccine with and without the B subunit of cholera toxin, Infect. Immun. 55 (1987) 1116–1120.
[18] M.M. Patel, D. Steele, J.R. Gentsch, J. Wecker, R.I. Glass, U.D. Parashar, Real-world impact of rotavirus vaccination, Pediatr. Infect. Dis. J. 30 (2011) S1–S5.
[19] A.M. Svennerholm, J. Tobias, Vaccines against enterotoxigenic *Escherichia coli*, Expert Rev. Vaccines 7 (2008) 795–804.
[20] A.M. Mowat, O.R. Millington, F.G. Chirdo, Anatomical and cellular basis of immunity and tolerance in the intestine, J. Pediatr. Gastroenterol. Nutr. 39 (Suppl. 3) (2004) S723–S724.
[21] S.J. Savarino, E.R. Hall, S. Bassily, F.M. Brown, F. Youssef, T.F. Wierzba, L. Peruski, N.A. El-Masry, M. Safwat, M. Rao, H. El Mohamady, R. Abu-Elyazeed, A. Naficy, A.M. Svennerholm, M. Jertborn, Y.J. Lee, J.D. Clemens, Oral, inactivated, whole cell enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine: results of the initial evaluation in children. PRIDE Study Group, J. Infect. Dis. 179 (1999) 107–114.
[22] J. Tobias, M. Lebens, I. Bolin, G. Wiklund, A.M. Svennerholm, Construction of non-toxic *Escherichia coli* and *Vibrio cholerae* strains expressing high and immunogenic levels of enterotoxigenic *E. coli* colonization factor I fimbriae, Vaccine 26 (2008) 743–752.
[23] S.D. Isidean, M.S. Riddle, S.J. Savarino, C.K. Porter, A systematic review of ETEC epidemiology focusing on colonization factor and toxin expression, Vaccine 29 (2011) 6167–6178.
[24] J. Holmgren, L. Bourgeois, N. Carlin, J. Clements, B. Gustafsson, A. Lundgren, E. Nygren, J. Tobias, R. Walker, A.M. Svennerholm, Development and preclinical evaluation of safety and immunogenicity of an oral ETEC vaccine containing inactivated *E. coli* bacteria overexpressing colonization factors CFA/I, CS3, CS5 and CS6 combined with a hybrid LT/CT B subunit antigen, administered alone and together with dmLT adjuvant, Vaccine 31 (2013) 2457–2464.
[25] A. Lundgren, S. Leach, J. Tobias, N. Carlin, B. Gustafsson, M. Jertborn, L. Bourgeois, R. Walker, J. Holmgren, A.M. Svennerholm, Clinical trial to evaluate safety and immunogenicity of an oral inactivated enterotoxigenic *Escherichia coli* prototype vaccine containing CFA/I overexpressing bacteria and recombinantly produced LTB/CTB hybrid protein, Vaccine 31 (2013) 1163–1170.
[26] C.J. Davitt, E.C. Lavelle, Delivery strategies to enhance oral vaccination against enteric infections, Adv. Drug Deliv. Rev. 91 (2015) 52–69.
[27] A.M. Mowat, W.W. Agace, Regional specialization within the intestinal immune system, Nat. Rev. Immunol. 14 (2014) 667–685.
[28] K.A. Woodrow, K.M. Bennett, D.D. Lo, Mucosal vaccine design and delivery, Annu. Rev. Biomed. Eng. 14 (2012) 17–46.
[29] D.E. Katz, A.J. DeLorimier, M.K. Wolf, E.R. Hall, F.J. Cassels, J.E. van Hamont, R.L. Newcomer, M.A. Davachi, D.N. Taylor, C.E. McQueen, Oral immunization of adult volunteers with microencapsulated enterotoxigenic *Escherichia coli* (ETEC) CS6 antigen, Vaccine 21 (2003) 341–346.
[30] K. Keohane, M. Rosa, I.S. Coulter, B.T. Griffin, Enhanced colonic delivery of ciclosporin A self-emulsifying drug delivery system encapsulated in coated minispheres, Drug Dev. Ind. Pharm. (2015) 1–9.
[31] M.M. Tambuwala, M.C. Manresa, E.P. Cummins, V. Aversa, I.S. Coulter, C.T. Taylor, Targeted delivery of the hydroxylase inhibitor DMOG provides enhanced efficacy with reduced systemic exposure in a murine model of colitis, J. Control. Release 217 (2015) 221–227.
[32] I. Coulter, B.F. McDonald, V. Aversa, M. Rosa, Formulations, 2014 (Google Patents).
[33] M. Villavedra, H. Carol, M. Hjulstrom, J. Holmgren, C. Czerkinsky, "PERFEXT": a direct method for quantitative assessment of cytokine production in vivo at the local level, Res. Immunol. 148 (1997) 257–266.
[34] R.G. Strickley, Solubilizing excipients in oral and injectable formulations, Pharm. Res. 21 (2004) 201–230.
[35] Y. Lopez, M. Pastor, J.F. Infante, D. Diaz, R. Oliva, S. Fernandez, B. Cedre, T. Hernandez, L. Campos, A. Esquisabel, J.L. Pedraz, V. Perez, A. Talavera, Repeated dose toxicity study of *Vibrio cholerae*-loaded gastro-resistant microparticles, J. Microencapsul. 31 (2014) 86–92.
[36] M.F. Pasetti, J.K. Simon, M.B. Sztein, M.M. Levine, Immunology of gut mucosal vaccines, Immunol. Rev. 239 (2011) 125–148.
[37] N. Lycke, Recent progress in mucosal vaccine development: potential and limitations, Nat. Rev. Immunol. 12 (2012) 592–605.
[38] D.P. Snider, The mucosal adjuvant activities of ADP-ribosylating bacterial enterotoxins, Crit. Rev. Immunol. 15 (1995) 317–348.
[39] A. Lundgren, L. Bourgeois, N. Carlin, J. Clements, B. Gustafsson, M. Hartford, J. Holmgren, M. Petzold, R. Walker, A.M. Svennerholm, 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 32 (2014) 7077–7084.
[40] E. Abautret-Daly, C.J. Davitt, E.C. Lavelle, Harnessing the antibacterial and immunological properties of mucosal-associated invariant T cells in the development of novel oral vaccines against enteric infections, Biochem. Pharmacol. 92 (2014) 173–183.
[41] V. Cerundolo, P. Barral, F.D. Batista, Synthetic iNKT cell-agonists as vaccine adjuvants—finding the balance, Curr. Opin. Immunol. 22 (2010) 417–424.
[42] L.J. Carreno, S.S. Kharkwal, S.A. Porcelli, Optimizing NKT cell ligands as vaccine adjuvants, Immunotherapy 6 (2014) 309–320.
[43] E. Nygren, J. Holmgren, S.R. Attridge, Murine antibody responses following systemic or mucosal immunization with viable or inactivated *Vibrio cholerae*, Vaccine 26 (2008) 6784–6790.
[44] D.M. Smith, J.K. Simon, J.R. Baker Jr., Applications of nanotechnology for immunology, Nat. Rev. Immunol. 13 (2013) 592–605.
[45] X. Zhao, M. Zhang, Z. Li, F.R. Frankel, Vaginal protection and immunity after oral immunization of mice with a novel vaccine strain of *Listeria monocytogenes* expressing human immunodeficiency virus type 1 gag, J. Virol. 80 (2006) 8880–8890.
[46] J.R. McGhee, H. Kiyono, S.M. Michalek, J. Mestecky, Enteric immunization reveals a T cell network for IgA responses and suggests that humans possess a common mucosal immune system, Antonie Van Leeuwenhoek 53 (1987) 537–543.
[47] A. Aase, H. Sommerfelt, L.B. Petersen, M. Bolstad, R.J. Cox, N. Langeland, A.B. Guttormsen, H. Steinsland, S. Skrede, P. Brandtzaeg, Salivary IgA from the sublingual compartment as a novel noninvasive proxy for intestinal immune induction, Mucosal Immunol. (2015), http://dx.doi.org/10.1038/mi.2015.107 [Epub ahead of print].
[48] M. Jertborn, A.M. Svennerholm, J. Holmgren, Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease, J. Clin. Microbiol. 24 (1986) 203–209.
[49] C. Ahren, M. Jertborn, A.M. Svennerholm, Intestinal immune responses to an inactivated oral enterotoxigenic *Escherichia coli* vaccine and associated immunoglobulin A responses in blood, Infect. Immun. 66 (1998) 3311–3316.
[50] E.J. Kunkel, C.H. Kim, N.H. Lazarus, M.A. Vierra, D. Soler, E.P. Bowman, E.C. Butcher, CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells, J. Clin. Invest. 111 (2003) 1001–1010.
[51] H.E. Mei, T. Yoshida, W. Sime, F. Hiepe, K. Thiele, R.A. Manz, A. Radbruch, T. Dorner, Blood-borne human plasma cells in steady state are derived from mucosal immune responses, Blood 113 (2009) 2461–2469.
[52] A. Huq, R.B. Sack, A. Nizam, I.M. Longini, G.B. Nair, A. Ali, J.G. Morris Jr., M.N. Khan, A.K. Siddique, M. Yunus, M.J. Albert, D.A. Sack, R.R. Colwell, Critical factors influencing the occurrence of *Vibrio cholerae* in the environment of Bangladesh, Appl. Environ. Microbiol. 71 (2005) 4645–4654.

[53] C.L. Fischer Walker, J. Perin, M.J. Aryee, C. Boschi-Pinto, R.E. Black, Diarrhea incidence in low- and middle-income countries in 1990 and 2010: a systematic review, BMC Public Health 12 (2012) 220.
[54] M.A. Adefisoye, A.I. Okoh, Identification and antimicrobial resistance prevalence of pathogenic Escherichia coli strains from treated wastewater effluents in Eastern Cape, South Africa, Microbiologyopen 5 (2016) 142–151.
[55] A.M. Svennerholm, From cholera to enterotoxigenic *Escherichia coli* (ETEC) vaccine development, Indian J. Med. Res. 133 (2011) 188–196.
[56] J. Tobias, A.M. Svennerholm, Strategies to overexpress enterotoxigenic *Escherichia coli* (ETEC) colonization factors for the construction of oral whole-cell inactivated ETEC vaccine candidates, Appl. Microbiol. Biotechnol. 93 (2012) 2291–2300.