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Oral vaccination using microdevices to deliver α-GalCer adjuvanted vaccine afford mucosal immunity

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Abstract

Oral vaccination has in the recent years gained a lot of attraction, mainly due to optimized patient compliance and logistics. However, the development of oral vaccines, especially oral subunit vaccines is challenging. Micro technology can be utilized to overcome some of these challenges, by facilitating protection and effective delivery of the vaccine components in the gastrointestinal tract (GI tract). One such technology is Microcontainers (MCs), which can be realized to be mucoadhesive and to target specific regions of the GI tract via oral delivery. Here, we test MCs, for oral delivery of the *C. Trachomatis* vaccine candidate CTH522, in combination with effective mucosal adjuvants. The adjuvants alpha-galactosylceramide (α-GalCer), C-di-GMP and cholera toxin B were compared *in vivo*, to identify the most prominent adjuvant for formulation with CTH522. Formulations were administered both purely oral and as boosters following a subcutaneous (s.c.) prime with CTH522 in combination with the CAF®01 adjuvant. CTH522 formulated with α-GalCer showed to be the most efficient combination for the oral vaccine, based on the immunological analysis. Lyophilized formulation of CTH522 and α-GalCer was loaded into MCs and these were subsequently coated with Eudragit L100-55 and evaluated *in vivo* in mice for the ability of MCs to mediate intestinal vaccine delivery and increase immunogenicity of the vaccine. Mice receiving oral prime and boosters did show a significantly enhanced mucosal immune responses compared to naive mice. This indicates the MCs are indeed capable of delivering the vaccine formulation intact and able to stimulate the immune cells. Mice orally boosted with MCs following a s.c. prime with CAF01, demonstrated improved systemic and local Th17 responses, along with increased local IFN-γ and IgA levels compared to both the s.c. prime alone and the homologous oral prime-boost immunization. However, due to the relatively weak observed effect of the MC delivery on the immune responses, it was hypothesized that the MCs are proportionally too large for the GI tract of mice, and thus cleared before an effective immune response can be induced. To investigate this, MCs were loaded with $BaSO_4$, and orally administered to mice. Analysis with X-ray and CT showed a transit time of approximately 1-1.5 h from the stomach to the cecum, corresponding to the standard transit time in mice, and an extremely narrow absorption window. This indicates that mice is not a suitable animal model for evaluation of MCs. These data should be taken into consideration in future *in vivo* trials with this and similar technologies, where larger animals might be a necessity for proof-of-concept studies.

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1. Introduction

In the recent years, requirements for vaccines targeting mucosal pathogens such as influenza- and corona viruses, have gained a high amount of interest, as these pathogens constitute a continual global threat [1]. This is not only the case with respiratory diseases, but also other pathogens like those causing sexual transmitted diseases (STDs) which likewise gain entry through the mucosa. One example is the gram-negative bacteria *Chlamydia Trachomatis* one of the most common sexually transmitted bacterial diseases with 129 million infected people annually [2]. Untreated or repeated bacterial infection with *C. Trachomatis* can lead to pelvic inflammatory disease ultimately causing tubal factor infertility and ectopic pregnancy. Recently it has become clear that following treatment for a chlamydial genital infection, woman cured of genital infection, often get re-infected due to autoinoculation from the lower gastrointestinal (GI) tract, because chlamydia has the ability to reside in the GI tract for long periods of time in the absence of clinical disease [3]–[5]. Therefore, a vaccine against infection with *C. trachomatis* could potentially benefit from inducing genital and intestinal mucosal- in addition to systemic immunity.

Nearly all modern vaccine research is based on subunit vaccines, due to ease of modification and high safety. These advantages, however, result in reduced immunogenicity, creating the essential need for the vaccines to be formulated with adjuvants. Today, a substantial amount of adjuvants exits, and their individual function varies according to the type and intended purpose, but also on factors such as, mode of administration and antigen formulation [4],[5]. A lead vaccine candidate against *C. Trachomatis* is the protein based subunit antigen CTH522, which requires co-delivery with an effective adjuvant that supports mucosal immunity [8]. The liposomal adjuvant CAF01 has showed promise in this regard, being able to stimulate Th1 and Th17 cells, needed for effective mucosal protection [9],[10]. In a recent clinical phase 1 trial, the CTH522 vaccine was evaluated by intramuscular injection in combination with CAF01, followed by intranasal administration with CTH522 alone. Here, the CAF01 adjuvanted CTH522 achieved a promising immunogenicity profile, generating neutralizing systemic and genital IgG and IgA antibodies [8]. Currently, the only subunit antigen in a licensed mucosal vaccine is the Cholera Toxin B (CTB), which has frequently been investigated as an adjuvant. CTB have been known to induce mucosal immune stimulation via various routes of administrations, such as intranasal, sublingual and oral [1],[8]. However, the immunomodulating effect of CTB is questioned by the presence of residual cholera toxin or LPS in CTB preparations, making it a challenge to separate adjuvanticity from toxicity [1]. Some recent studies have deemed highly purified CTB as an inducer of immune tolerance, rather than a promoter of mucosal immune responses when administered orally or intranasally [12]. Another mucosal adjuvant candidate is Cyclic-di-GMP (c-di-GMP), a ligand of the STING pathway. Intranasal administered c-di-GMP has on several occasions been shown to develop protection in the respiratory tract, primarily observed to induce high Th1 and Th17 stimulation. Two studies using c-di-GMP as an adjuvant in an influenza vaccine, recorded high stimulation of Th1 IFN-genes and enhanced Th17 cytokine production, as a feat of activating STING [13], [14]. *Madhun et al.* also investigated c-di-GMP as an intramuscular adjuvant, which interestingly showed no enhancement of the immune response, compared to administration with only the antigen. Furthermore, the glycolipid α-Galactosylceramide (α-GalCer), an activator of invariant Natural Killer T (iNKT) cells, have mostly been used in cancer immunotherapy [15]. However, α-GalCer has been reevaluated as a promising adjuvant of the mucosal immune system, especially following oral administration. In recent studies, whole-cell killed *Helicobacter Pylori* and enterotoxigenic *Escherichia coli* vaccines adjuvanted with α-GalCer, demonstrated induction of intestinal IgA and Th1 cell immunity, along with serum IgG responses following oral administration [13],[14].

Oral delivery of vaccines holds potential in lowering the cost of administration and logistics of vaccine dosing significantly, especially in mass vaccination situations. In addition, oral administration could

encourage vaccination for the population due to the high patient compliance [18]. Oral immunization draws its advantage in being able to induce effective secretory IgA antibody and T-cell responses in mucosal tissues in the gastrointestinal (GI) tract, and other local mucosal sites [19]. Oral delivery of subunit vaccines, such as the CTH522 vaccine candidate, is however very sensitive to chemical and enzymatic degradation in the stomach and intestines [18]. Thus, subunit vaccines are in need of innovative oral delivery systems in addition to potent adjuvants. This system should be capable of protecting the vaccine from degradation and facilitate uptake by effective delivery to the intestinal epithelium. The mucosal tissue in the intestines, contains an abundance of immune cells to be utilized, making it a noteworthy target [20]. The concept has been widely studied, and many attempts have been made to facilitate effective oral delivery of vaccines. This includes technologies such as nano- and micro-particular systems such as PEG and PLGA particles, along with permeation enhancers for vaccine formulations such as ionic liquids [21], [22], [23]. Previously, we have tested microcontainers (MCs) as a delivery system of a spray dried vaccine formulation consisting of cubosomes, the adjuvant Quil-A and the model antigen ovalbumin (OVA) [24]. It was observed that the MCs are indeed capable of protecting their content through the GI tract, until intended release in the small intestine of mice by the pH dependent polymeric coating Eudragit L100-55 (EL100-55). Furthermore, it was shown that the humoral response could be slightly improved with oral boosters after a parental prime. However, the MCs ability to deliver an antigen candidate, targeting a mucosal infecting pathogen along with promising mucosal adjuvants, remains to be tested.

In this study, we investigated the mucosal immune inducing adjuvants, CTB, c-di-GMP and α-GalCer for the purpose of oral vaccination with the *C. Trachomatis* antigen CTH522, to boost and redirect a subcutaneous (s.c.) prime injection with CTH522 + CAF01 into the intestines. Moreover, a lyophilization procedure was developed and optimized for the vaccine formulations to enhance thermal stability. In previous studies, MCs have been coated with poly(lactic-co-glycolic acid) (PLGA) or chitosan for drug delivery purposes. We here tested their capability to both function as mucosal agents and coatings on MCs, along with the polymer EL100-55, in an *in vivo* comparison study. Findings of the most promising adjuvant along with the most optimal MC coating are combined, and their ability to induce a mucosal immune response against CTH522 via oral administration in mice is evaluated (Fig. 1)**.** The capabilities of the vaccine formulations and micro technology to induce immune responses, were evaluated by analysis of cytokines elicited from T-cells along with IgG and IgA antibodies produced by B-cells.

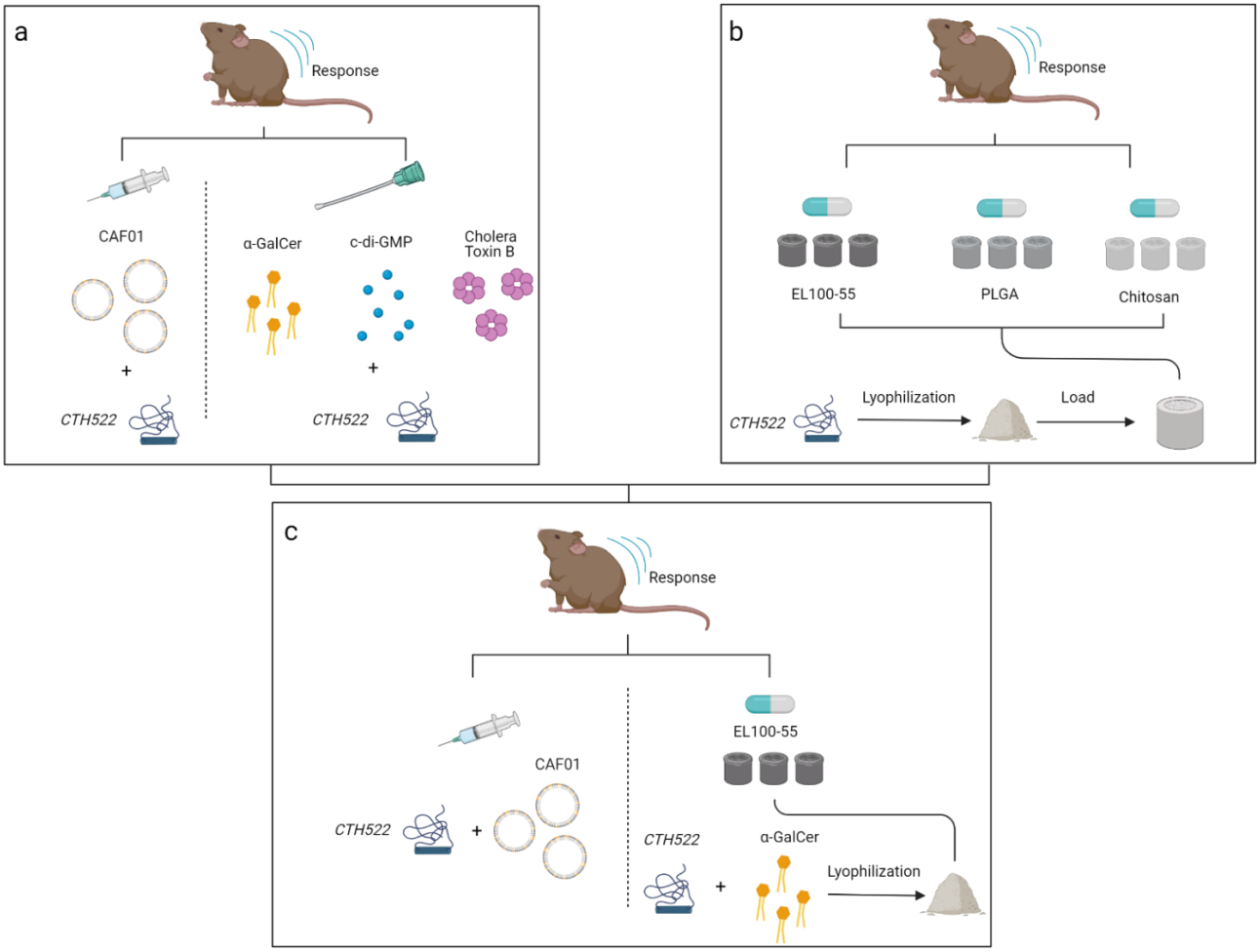

Fig. 1. Overview of the work flow conducted in this paper. To identify the most effective mucosal adjuvant, an *in vivo* screening of the adjuvants α-GalCer, c-di-GMP and Cholera Toxin B, formulated with CTH522 was set up (a). Formulations were administered orally with gavage either following an s.c. prime with CAF01 + CTH522 or as oral administration only. To enhance thermal stability of the vaccine formulation, a lyophilization procedure of CTH522 was implemented (b). Lyophilized powder was loaded into microcontainers (MCs) and subsequently coated with the polymers Eudragit L100-55 (EL100-55), poly(lactic-co-glycolic acid) (PLGA) or chitosan and filled into gelatin capsules for oral immunization to mice, following an s.c. prime with CAF01 + CTH522 (b). Based on the results from these experiments, an experiment with lyophilized CTH522 adjuvanted with α-GalCer, loaded into MCs and coated with EL100-55 was conducted (c). MCs were administered orally either following an s.c. prime with CAF01 + CTH522 or as oral administration only. *Created with Biorender.com*

2. Materials and methods

2.1 Materials

C-di-GMP was purchased from Invivogen (San Diego, CA, USA), CTB was obtained from Sigma Aldrich (St. Louis, MO, USA) and α-Galactosylceramide was purchased from Avanti lipids (KRN7000, Bermingham, AL, USA). CTH522 and CAF01 were produced in-house as described in[8]. PLGA (low MW 7–17 kDa, 50:50 PLA:PGA), trehalose, L-histidine and soybean trypsin inhibitor were obtained from Sigma-Aldrich (St. Louis, MO, USA). Eudragit L100-55 was purchased from Evonik (Darmstadt, Germany). Size M gelatin capsules were bought from Torpac (SG Heerlen, The Netherlands). PBS and non-essential amino acids were obtained from Life Technologies (Roskilde Denmark). HEPES buffer, RPMI 1640, L-Glutamine and sodium pyruvate was purchased from Invitrogen (Weltham, MA, USA). Fetal calf serum was from Biowest (Nuaillé, France), TMB ready-to-use-substrate was bought from Kem-En-Tec (Taastrup, Denmark), Tween20 was from Merck (Darmstadt, Germany), H_2SO_4 and $BaSO_4$ (precipitated, 99%) was bought from VWR International (Radnor, PA, USA). NUNC 96 well Maxisorp plates was obtained from Thermo Scientific (Roskilde, Denmark) and Falcon 100µm nylon cell strainers were bought from Corning (Vordingborg, Denmark). HRP rabbit anti-mouse IgG was purchased from AH Diagnostics (Tilst, Denmark), Biotin goat anti-mouse IgA was obtained from Sourthern Biotech (Bermingham, AL, USA) and Streptavidin – HRP conjugate was bought from BD Pharmingen (Lyngby, Denmark). Biotin rat anti-mouse IFN-γ and purified rat anti-mouse IFN-γ was purchased from BD Pharmingen (Lyngby, Denmark). Biotin anti-mouse IL-17A and purified rat anti-mouse IL-17A was obtained from BioLegend (San Diego, CA, USA). Concavalin A was purchased from GE Healthcare (Marlborough, MA, USA). CB6F1 mice were bought from Envigo (Indianapolis, IN, USA)

2.2 Mice

Male CB6F1 mice were purchased weighing at least 25 g (approx. 12 weeks old) following the minimum oral dosing recommendations from Torpac. Mice were acclimatized 1 week prior to starting experiments. Mice had free access to food and water at all times. All experiments were approved by the Animal Experiments Inspectorate of Denmark, under the license 2020-15-0201-00610 and were conducted in compliance with the Danish laws regulating experiments on animals and the EC Directive 2010/63/EU.

2.3 CTH522 formulation with CAF01, C-di-GMP, CTB and α-GalCer

CTH522 (1.4 mg/mL) were thoroughly vortexed with 1 mg/mL CAF01 every 10 min for 30 min prior to immunization. CTB was rehydrated to a concentration of 5 mg/mL and mixed with CTH522 by pipetting. C-di-GMP was similarly rehydrated to a concentration of 5 mg/mL and mixed with CTH522 by pipetting. α-GalCer was mixed, as suggested by Avanti, in 5.7% trehalose, 0.75% L-histidine, and 0.5% Tween 20, making a 5 mg/mL solution. After mixing, the solution was heated at 80 ℃ and sonicated every 10 min for 1 min, until the material was completely dissolved. Dissolved α-GalCer was then mixed with CTH522 and thoroughly vortexed.

2.4 Lyophilization of vaccine formulation

CTH522 alone or adjuvanted with α-GalCer were lyophilized in a Christ Delta 2-24 LSCplus freeze-dryer (Christ, Osterode am Harz, Germany) with the program depicted in Table 1. Antigen and adjuvants were lyophilized in a 10% trehalose + 10 mM Tris-base formulation.

Table 1. The parameters for the program used to lyophilize the CTH522 + α-GalCer vaccine formulation.

Phase	Freeze	Primary Drying	Primary Drying	Primary Drying	Secondary Drying	Secondary Drying
Time	3:00 h	0:15 h	0:15 h	36:00 h	5:00 h	10:00 h
Temp.	-42 °C	-42 °C	-30 °C	-30 °C	20 °C	20 °C
Vacuum	-	0.120 mbar	0.120 mbar	0.120 mbar	0.120 mbar	0.120 mbar

2.5 SDS-gel of hydrated vaccine formulation

Gel electrophoresis was performed in a Mini-PROTEAN Tetra system (Bio Rad, Hercules, CA, USA) with a Mini-PROTEAN TGX Precast 12 well gel (Bio Rad, Hercules, CA, USA). For the denatured proteins, 2 µg of rehydrated lyophilized CTH522, and 2 µg non-lyophilized CTH522 were formulated with sample buffer Tris/Glycerol, Bromphenol Blue) + SDS and DTT in a 1:1 ratio and loaded on the gel. For the native proteins 2 µg of rehydrated lyophilized CTH522, and 2 µg non-lyophilized CTH522 were formulated with sample buffer ÷ SDS and DTT in a 1:1 ratio and loaded on the gel. The gel was run at 300 V for 16 min using Power Pac 300 (Bio Rad, Hercules, CA, USA). The gel was removed and washed with deionized water and then emerged in Bio-Safe Coomassie G250 Stain (Bio Rad, Hercules, CA, USA) for 60 min, followed by wash with dionized water for 2x30 min.

2.6 Fabrication, drug loading, polymeric coating and capsule filling of microcontainers

MCs were fabricated with the negative epoxy photoresist SU-8 by a two-step photolithography process as described previously [25]. The MCs were produced on top of a titanium|gold (Ti|Au) coated silicon wafer to allow easy removal from the wafer. The wafer was then cut into 12.8 by 12.8 mm^2 chips containing 25 by 25 arrays of MCs using a dicing saw (DISCO, München, Germany). MCs on chips were loaded with vaccine formulation powder of CTH522 + α-GalCer using an embossing method as described previously [26]. A shadow mask was used to cover the gaps between the MCs, thus, filling the MCs without filling the space between them with powder. The average powder load in the MCs was estimated by weighing 10 chips before and after loading and calculating the average of 1 microcontainer. After loading, the MCs were sealed with either EL100–55, PLGA or chitosan through a spray coating process, using an ExactaCoat spray coater (Sono Tek, Milton, Canada) equipped with an ultrasonic nozzle actuated at 120 kHz (Accumist, Sono Tek, Milton, Canada). Acetic acid containing 0.5%(w/w) chitosan (low molecular weight, 75-85% deacetylated) was sprayed with the spray coating parameters for chitosan depicted in Table 2. Dichloromethane (DCM) containing 0.5%(w/w) PLGA (7-17 kDa, 50:50 PLA:PGA) was sprayed with the spray coating parameters for PLGA depicted in Table 2. Isopropanol containing 1% (w/v) EL100-55 and 5% (w/w in relation to EL100–55) dibutyl sebacate was sprayed with the spray coating parameters for EL100-55 depicted in Table 2.

Table 2. Spray coating parameters used for the coating of the polymers chitosan, PLGA and EL100-55 on microcontainers (MCs).

	Feed flow	Generator power	Air pressure	Temperature	Nozzel distance to MCs	Speed	Passages
Chitosan	0.1 mL/min	1.3 W	0.030 kPa	50 °C	7.5 cm	25 mm/s	110
PLGA	0.1 mL/min	2.2 W	0.030 kPa	Room temp.	5 cm	10 mm/s	55
EL100-55	0.1 mL/min	2.2 W	0.028 kPa	35 °C	5 cm	10 mm/s	25

After the coating procedure, MCs were directly removed from the chips using a scalpel. MCs were filled into size M gelatin capsules using a size M funnel (Torpac, SG Heerlen, The Netherlands). The amount of MCs in the capsules were determined by weighing the capsules before and after filling. Based on the filling of 10 capsules, a size M capsule could contain 82 ± 3.7 MCs.

2.7 Microcontainer coating characterization and release study

Microcontainer chips were placed in acid baths subsequent to loading with lyophilized CTH522 formulation and coated with chitosan, PLGA or EL100-55. Chips was submerged in 36.6 °C 2 mM pH 4.7 maleic acid for 60 min and visualized with a table top scanning electron microscope (SEM) (Hitachi TM3030plus, Tokyo, Japan), using 15 kV acceleration voltage at 40× and 50× magnification. Chips were then submerged in 36.6 °C 10 mM pH 6.6 maleic acid for 30 min and 60 min, and visualized after each time point with SEM using the same settings as described above.

2.8 *In vivo* studies

Four *in vivo* studies were conducted in this paper (Table 3, supporting information). Mice were immunized according to Table 3, and dosed either s.c. or orally with gavage or MCs. MCs were administered in size M gelatin capsules. Mice were euthanized and harvested on day 56.

Table 3. Scheme of the setup for the four *in vivo* studies conducted in this paper, depicting formulation, dosage amount and administration form on the immunization days of the individual groups in each of the studies. Coating of microcontainers (MCs) is included for the groups, which were administered orally with them. Prime immunization is set at day 0, first booster immunization was set at day 21 and second booster immunization was set at day 42. Naive groups were not dosed with anything.

In vivo study nr.	Group nr.	Prime (day 0)	1. Booster (day 21)	2. Booster (day 42)	MC coating
1	1	Naive	-	-	-
	2	5 µg CTH522 + 5 µg CAF01 (s.c.)	-	-	-
	3.	5 µg CTH522 + 5 µg CAF01 (s.c.)	10 µg CTH522 + 10 µg c-di-GMP (oral gavage)	10 µg CTH522 + 10 µg c-di-GMP (oral gavage)	-
	4	5 µg CTH522 + 5 µg CAF01 (s.c.)	10 µg CTH522 + 10 µg α-GalCer (oral gavage)	10 µg CTH522 + 10 µg α-GalCer (oral gavage)	-
	5	5 µg CTH522 + 5 µg CAF01 (s.c.)	10 µg CTH522 + 10 µg CTB (oral gavage)	10 µg CTH522 + 10 µg CTB (oral gavage)	-
	6	10 µg CTH522 + 10 µg c-di-GMP (oral gavage)	10 µg CTH522 + 10 µg c-di-GMP (oral gavage)	10 µg CTH522 + 10 µg c-di-GMP (oral gavage)	-
	7	10 µg CTH522 + 10 µg α-GalCer (oral gavage)	10 µg CTH522 + 10 µg α-GalCer (oral gavage)	10 µg CTH522 + 10 µg α-GalCer (oral gavage)	-
	8	10 µg CTH522 + 10 µg CTB (oral gavage)	10 µg CTH522 + 10 µg CTB (oral gavage)	10 µg CTH522 + 10 µg CTB (oral gavage)	-
2	1	Naive	-	-	-
	2	Lyophilized 5 µg CTH522 + 5 µg α-GalCer (s.c.)	Lyophilized 5 µg CTH522 + 5 µg α-GalCer (s.c.)	-	-
	3	5 µg CTH522 + 5 µg α-GalCer (s.c.)	5 µg CTH522 + 5 µg α-GalCer (s.c.)	-	-
3	1	Naive	-	-	-
	2	5 µg CTH522 + 5 µg CAF01 (s.c.)	-	-	-
	3	5 µg CTH522 + 5 µg CAF01 (s.c.)	10 µg CTH522 (oral MCs)	10 µg CTH522 (oral MCs)	EL100-55
	4	5 µg CTH522 + 5 µg CAF01 (s.c.)	10 µg CTH522 (oral MCs)	10 µg CTH522 (oral MCs)	PLGA
	5	5 µg CTH522 + 5 µg CAF01 (s.c.)	10 µg CTH522 (oral MCs)	10 µg CTH522 (oral MCs)	Chitosan
4	1	Naive	-	-	-
	2	5 µg CTH522 + 5 µg CAF01 (s.c.)	-	-	-
	3	5 µg CTH522 + 5 µg CAF01 (s.c.)	10 µg CTH522 + 10 µg α-GalCer (oral gavage)	10 µg CTH522 + 10 µg α-GalCer (oral gavage)	-
	4	5 µg CTH522 + 5 µg CAF01 (s.c.)	10 µg CTH522 + 10 µg α-GalCer (oral MCs)	10 µg CTH522 + 10 µg α-GalCer (oral MCs)	EL100-55
	5	10 µg CTH522 + 10 µg α-GalCer (oral MCs)	10 µg CTH522 + 10 µg α-GalCer (oral MCs)	10 µg CTH522 + 10 µg α-GalCer (oral MCs)	EL100-55

2.9 Sample and organ preparation for ELISA analysis

Whole blood was separated by centrifugation at 10.000 G for 10 min. Serum was removed and stored in 96 well plates. Fecal pellets were collected in cold feces buffer (PBS + 0.1 mg/mL Soybean trypsin inhibitor + 1% w/v BSA + 25 mM EDTA + 50% v/v glycerol + 1mM PMSF). Pellets were then broken to form a suspension and incubated for 4 h on ice. Solids were separated from liquid by centrifugation for 10 min at 15.500 G at 4 °C. Supernatants were transferred to microfuge tubes, and blocked over night with PBS + 1% (w/v) BSA. Isolated spleens and PPs were filtered through a cell strainer, suspended in RPMI media without FBS and centrifuged for 5 min at 1800 rpm. Then resuspended in 1mL using complete RPMI (cRPMI) (supplemented with HEPES, penicillin–streptomycin, sodium pyruvate, l-glutamine and non-essential amino acids) with 10% FBS (v/v) and counted on a NucleoCounter NC-200 (Chemometec, Allerød, Denmark). Cell cultures were seeded (2×10^5/well) and stimulated with the CTH522 (1.4 µg/mL); media cRPMI (as negative control) and concavalin A (1 µg/mL) (as positive control). The supernatants were harvested after 72 h incubation and stored at -20 °C.

2.10 ELISA

For the IgG antibody ELISA, 96 well plates were coated with CTH522 and then blocked with 2% w/v BSA in PBS. Sera were diluted 1:100 and 3-fold diluted across well plates. Samples were then incubated for 2 h with HRP-conjugated IgG anti-mouse monoclonal antibody. For IgA antibody ELISA, wells were coated with CTH522 and then blocked with 2% skim milk in PBS. Fecal supernatants or sera were loaded on well plates and titrated in a 2-fold dilution across the plates and detection was done using biotin conjugated anti-mouse IgA (Southern Biotech) for 1 h followed by streptavidin-HRP (BD Biosciences) for 30 min. IgG and IgA were color developed using TMB. Development was stopped with 0.2M H_2SO_4 after 10 min and absorbance was measured at 450 nm (correction at 570/620 nm) using a Polarstar Omega Microplate Reader (BMG Labtech). The absorbance values were plotted as a function of the reciprocal dilution of serum samples. Antibody titers were determined as the highest serum dilution corresponding to a cut-off of ≥0.2 OD450 (supporting information). For the cytokine ELISA, 96 well plates were coated with purified anti-mouse IFN-γ or IL-17A in PBS at 4°C overnight. Free binding sites were blocked with 1% BSA. Spleen and PP culture supernatants were tested in triplicates, and detection was done by biotin-labeled rat anti-mouse IFN-γ or IL-17A. Samples were then incubated for 30 min with Streptavidin HRP. Color was developed and measured as described above. Standards of IFN-γ and IL-17A were used to determine the amount of cytokine in the samples.

2.11 CT-scanning and X-ray imaging of mice

To study the transit time of MCs in mice, MCs were loaded with the contrast agent $BaSO_4$ and coated with PLGA as described in 2.8. MCs were given to mice orally in size M gelatin capsules. Mice were anesthetized at time points 0.5 h, 1 h, 1.5 h and 2 h. Mice were frozen and then visualized with 3D visualizations made by CT scanning (Nikon XT H 225, Nikon Metrology, Tokyo, Japan). X-rays were generated using a voltage of 70 kV and a power of 30 W (current of 0.43 mA). The 3D visualizations were created from single planar scans using 1572 projections with 2 frames per projection and an exposure time of 0.5 s. Within the final scan time of approximately 27 min, the frozen mice did not seem to thaw. The voxel size, which corresponds to the spatial resolution, was kept constant at 114.41 µm by having the same distance between the x-ray probe and the sample for all scans even though the size of the frozen mice varied slightly. The following reconstruction was made in the software provided with the CT scanner system (CT Pro 3D, Nikon Metrology, Tokyo, Japan) using a Feldkamp, Davis and Kress filtered back-projection algorithm [27]. As a final step, a 3D visualization and analysis software (Avizo, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for the CT scan data to be processed and investigated. Subsequently, the GI tract were isolated from the mice for further analysis with X-ray imaging. Planar X-ray imaging of MCs loaded with $BaSO_4$ in the removed GI tracts was carried out, using a CT scanner, to obtain quantitative

information about their location. The distance between the x-ray probe and the samples was adjusted to get a magnification of 4 and X-rays were generated using a voltage of 70 kV and a power of 30 W (current of 0.43 mA). Image acquisition with 8 frames and an exposure time of 1 s was used for the planar X-ray images and a background signal for shading correction. The shading corrections and subsequent manually counting of the MCs loaded with $BaSO_4$ throughout the entire GI tracts were made using an image processing software (ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA).

2.12 Statistics

GraphPad Prism 9 software (San Diego, CA, USA) was used for data handling, analysis, and graphic representation. Statistical analysis was performed using the Kruskal–Wallis test followed by a post-hoc Dunn's multiple comparison test, where p-values below 5% were considered significant. Data is presented as mean ± standard error of the mean (SEM).

3 Results and discussion

3.1 Screening of mucosal adjuvants for formulation with CTH522 and oral administration

Adjuvants are necessary to induce a proper and efficient immune response against infectious pathogens, especially in regard to subunit type antigens. However, studies have shown that the function of an adjuvant can vary depending on how it is administrated and which kind of antigen is included [7], [28]. In this study, the adjuvants were thus tested according to how well they performed being administered orally in formulation with CTH522. CTH522 was formulated with c-di-GMP, α-GalCer or CTB and dosed orally to mice, either receiving oral prime and boosters, or as boosters following a subcutaneous (s.c.) prime with CTH522 adjuvanted with CAF01 (Fig. 2). In order to evaluate the ability of the oral administered adjuvant formulations to enhance immune responses, a naive group and a group receiving only s.c. prime of CTH522 and CAF01 were included as controls. The IgG specific antibodies in the serum, along with the cytokines elicited in the spleen, were measured as representatives of the systemic response (Fig. 2a, 2b, 2e). A trend towards higher levels of IFN-γ and IL-17A, indicators of Th1 and Th17 induction, was seen in all groups receiving oral gavage boosters compared to the s.c. priming group (Fig. 2a, 2b). Notably, α- GalCer did achieve higher levels of cytokine secretion compared to the other adjuvants, although not significantly. Low to inconsistent levels of IFN-γ and IL-17A comparable to the naive mice, were measured in the groups receiving oral prime and boosters. The CTH522 IgG specific titers, showed no significant difference between the s.c. group and the oral boosted groups (Fig. 2e), suggesting the measured IgG response is developed on account of the s.c. prime injection. Further evidence of this was seen in titers elicited by the oral dosed groups, which, like the cytokine analysis of these groups, were comparable to the naive mice. Cytokine levels in Peyer's Patches (PPs) along with CTH522 specific IgA antibody titers in feces were analyzed in order to represent the local mucosal immune response elicited in the intestine (Fig. 2c, 2d, 2f). Here, the same pattern in cytokine levels was observed as in the systemic response analysis. Mice receiving s.c. prime and oral boosters showed increased secretion of IFN-γ and IL-17A in PPs compared to the s.c. group, however not significantly (Fig. 2c, 2d). α-GalCer excelled slightly in stimulation of Th17 cells in the PPs compared to the other adjuvants. A change was however seen in the oral primed and boosted groups, where α-GalCer did achieve significantly higher levels of IFN-γ compared to the naive group ($p < 0.0286$, Fig 2c). Measured CTH522 specific IgA titers in fecal pellets showed no difference between the s.c. primed groups, suggesting that the response is an effect of the CAF01 injection (Fig. 2f). However, all groups receiving oral prime and boosters generated significantly higher IgA titers compared to the naive mice ($p = 0.0346$, Fig. 2f), indicating the adjuvants are capable of eliciting local IgA response when administered orally and in formulation with CTH522. The results observed in this study, points towards α-GalCer, as the most prominent adjuvant for formulation and oral administration with CTH522. A reason for this could be that being a glycolipid, α-GalCer is more stable and less prone to

immediate degradation by the low pH in the stomach. α-GalCer have also before shown to boost immunogenicity of HIV antigens peptide by simple oral immunization[29].

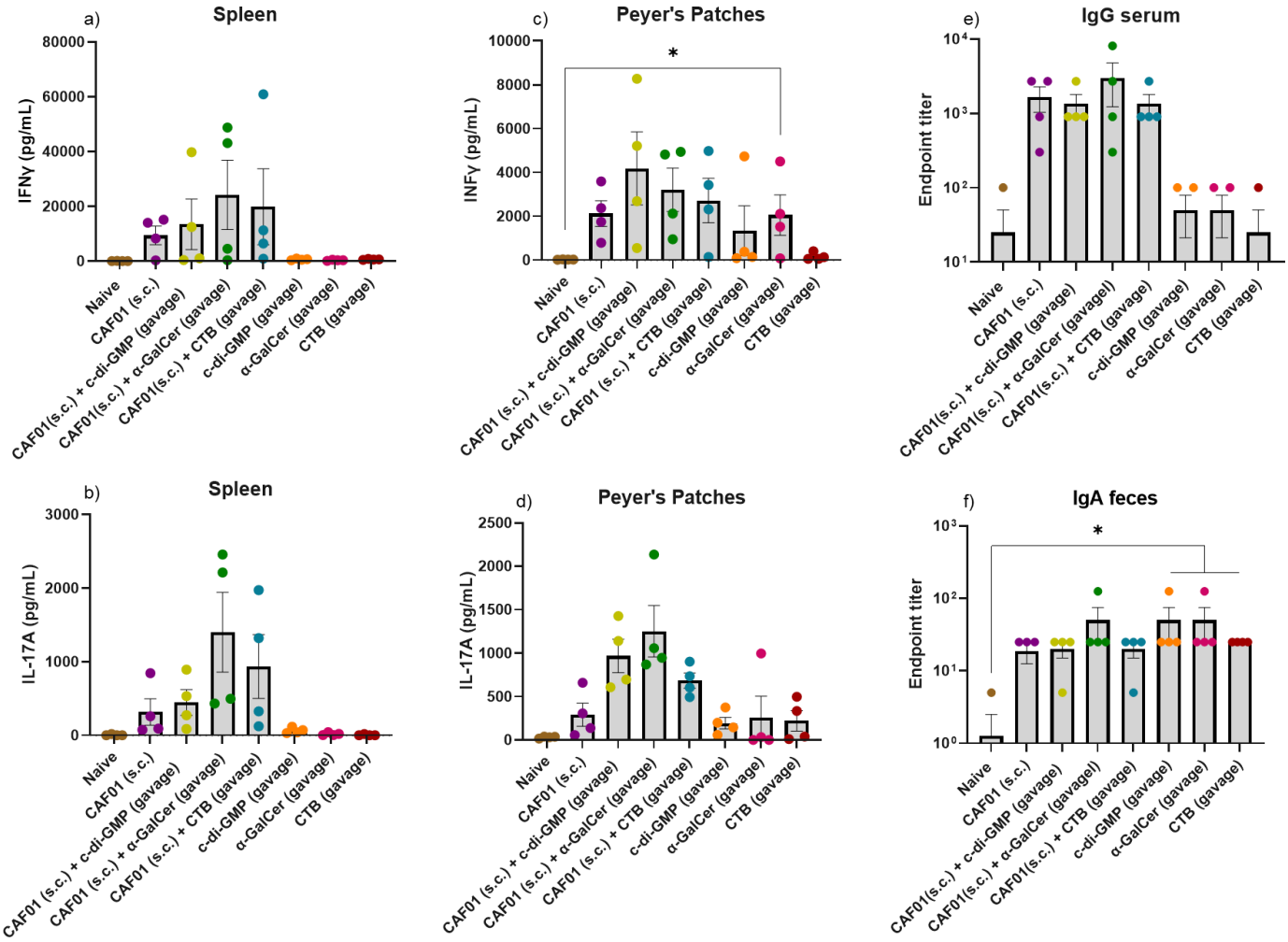

Fig. 2. Levels of secreted IFN-γ and IL-17A measured in spleen (a, b) and Peyer's Patches (c, d). CTH522 specific serum IgG and fecal IgA antibody titers (e, f). Mice, except the naive and CAF01(s.c.) group, were immunized 3 times in a prime-booster-booster regime, with either a sub cutaneous (s.c.) prime injection followed by oral boosters with gavage or oral prime followed by oral boosters with gavage. Immunizations were given at day 0, 21 and 42. Data are shown from individual mice and bars represent mean ± SEM (n = 4). * $p < 0.05$.

3.2 Lyophilization procedure for the CTH522 + α-GalCer formulation.

Since vaccine formulations are often thermally labile, lyophilization is frequently used to stabilize vaccine for distribution and storage [30]. This is especially of advantage in places where cold chain management is difficult. During the lyophilization process, solvent is removed, and replaced by a stabilizing substance which also functions as a lyoprotectant[30]. The method is complex and requires optimization of both process parameters and buffers, to achieve optimal drying of the formulation and no loss of immunogenicity in the case of vaccines[31]. The lyophilization program described in 2.4 was used with various excipients at different ratios, and evaluated by the consistency and visual appearance in reference to [32] (Table 4). 10%(w/v) Trehalose + 10 mM Tris were deemed the most prominent excipient and additive, achieving the best dried state post-lyophilization.

Table 4. Buffer formulations for optimization of lyophilization procedure. Post-lyophilization visual attributes were referenced to [32] and the consistency score were based on the ease of loading the powder into microcontainers-

Antigen	Adjuvant	Stabilizer	Buffer	Visual attribute of sample	Consistency score
CTH522 (10 µg)	α-GalCer (10 µg)	5% Trehalose	10 mM Tris	Uniform	+
			10 mM Tris + 2% Glycerol	Meltback	-
			PBS	Cracked	+
		10% Trehalose	10 mM Tris	Uniform	+++
			10 mM Tris + 2%Glycerol	Meltback	-
			PBS	Uniform	+
		15% Trehalose	10 mM Tris	Uniform	++
			10 mM Tris + 2%Glycerol	Meltback	-
			PBS	Uniform	++
		10% Sucrose	10 mM Tris	Uniform	++
			PBS	Cracked	+
		10% Lysine	10 mM Tris	Collapse	-
			PBS	Collapse	-
		10% Mannitol	10 mM Tris	Cracked	+
			10 mM Tris + 2% Glycerol	Meltback	-

Lyophilized CTH522 was then rehydrated and analyzed by SDS-PAGE to determine that no degradation was happening as a result of the lyophilization or rehydration process (Fig. 3a). To investigate if the lyophilization caused any loss of immunogenicity to the antigen, the formulation was evaluated in an *in vivo* study. Lyophilized CTH522 + α-GalCer was rehydrated and given s.c. to mice in a prime-booster regime (Fig. 3b, 3c, 3d). As controls, a naive group was included along with a group receiving the same formulation but non-lyophilized. From the study, it was evident that no loss of immunogenicity was seen, as no difference was observed between the lyophilized group and non-lyophilized group on any of the measured antibody or cytokine responses. The developed lyophilization process and optimized buffer formulation are thus capable to effectively lyophilize CTH522 while in formulation with α-GalCer, without causing damage to the antigen and successfully retain the immunogenicity. Antigens and adjuvants are usually not lyophilized while in formulation, as it can have implications on the process [33]. The developed protocol could potentially be used for other such formulations in future studies. It should be noted that optimization of the procedure would be necessary in any case, and the presented protocol would likely be most effective for antigens of related conformation. For example, could it be assumed that DNA, RNA and more complex antigens will not be compatible with the developed lyophilization protocol in this study.

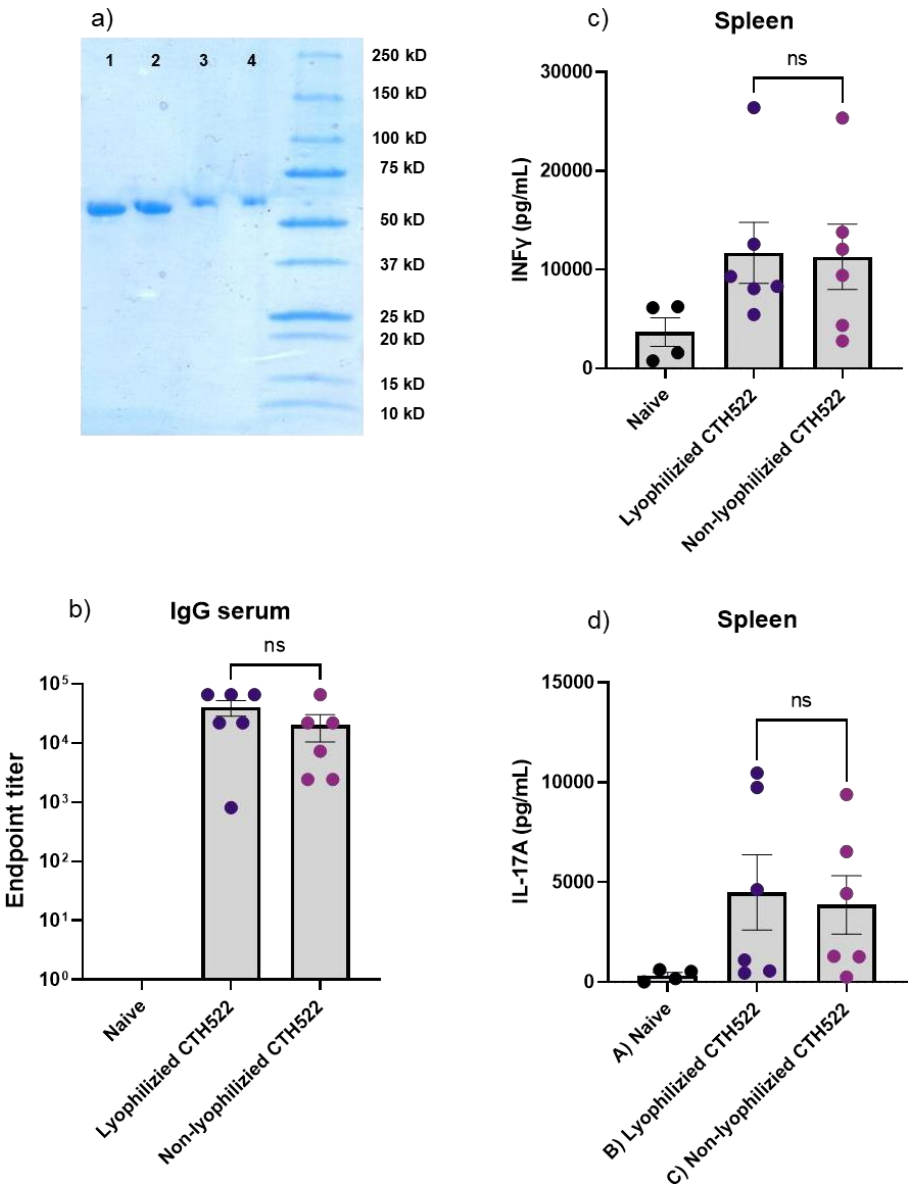

Fig. 3. SDS-gel of rehydrated lyophilized CTH522 + SDS (row 1), non-lyophilized CTH522 + SDS (row 2), rehydrated lyophilized CTH522 ÷ SDS (row 3) and non-lyophilized CTH522 ÷ SDS (row 4) (a). CTH522 specific serum IgG antibody titers (b). Levels of secreted IFN-γ and IL-17A measured in spleen (c, d). Mice, except the naive group, were immunized subcutaneously 2 times in a prime-booster regime at day 0 and 21. Data are shown from individual mice and bars represent mean ± SEM (n = 6).

3.3 Fabrication, loading and coating of microcontainers

MCs were fabricated with an outer diameter of 313.4 ± 1.7 µm and height of 289.4 ± 5.3 µm, and with an inner diameter of 262.5 ± 0.9 µm and height of 235.8 ± 4.4 µm. MCs were loaded with 3.1 ± 0.6 µg/microcontainer of powder and then sealed with either chitosan, PLGA or EL100–55 lids. The average thickness of the lid coating was measured by contact profilometry to be 27.3 ± 2.1 µm for chitosan, 28.7 ± 4.5 µm for PLGA and 25.7 ± 1.5 µm for EL100-55.

PLGA and chitosan have both been documented to have adjuvant properties and are widely used for mucosal delivery of antigens and active pharmaceutical ingredients (APIs), often as particle carriers [13],

[34], [35]. Here, it was investigated if coatings with PLGA or Chitosan on MCs could benefit the delivery of the CTH522 antigen. Additionally, the pH-dependent EL100-55 coating was also included. This polymer has the ability to stay solid at pH 4.7, corresponding to the pH in the stomach of mice, and dissolve at pH 6.6 corresponding to the pH in the small intestine of mice [36], [37]. These properties can be used to protect the content in the stomach and effectively target the small intestine for the release [24]. This was compared to naive mice and a group only receiving a s.c. prime to distinguish the oral response as in 3.1 (Fig. 4). Additionally, the local immune response in the intestine was also investigated for all three coatings, which have not been done before. Systemically no difference in response was seen (Fig. 4a, 4b, 4e), however, CTH522 specific IgA antibody levels measured in feces from MCs coated with EL100-55 were significantly increased ($p = 0.0488$, Fig. 4f). This suggests that the EL100-55 coating can be used for effective delivery of CTH522 with MCs, and that no improvement is gained from coatings with PLGA and chitosan.

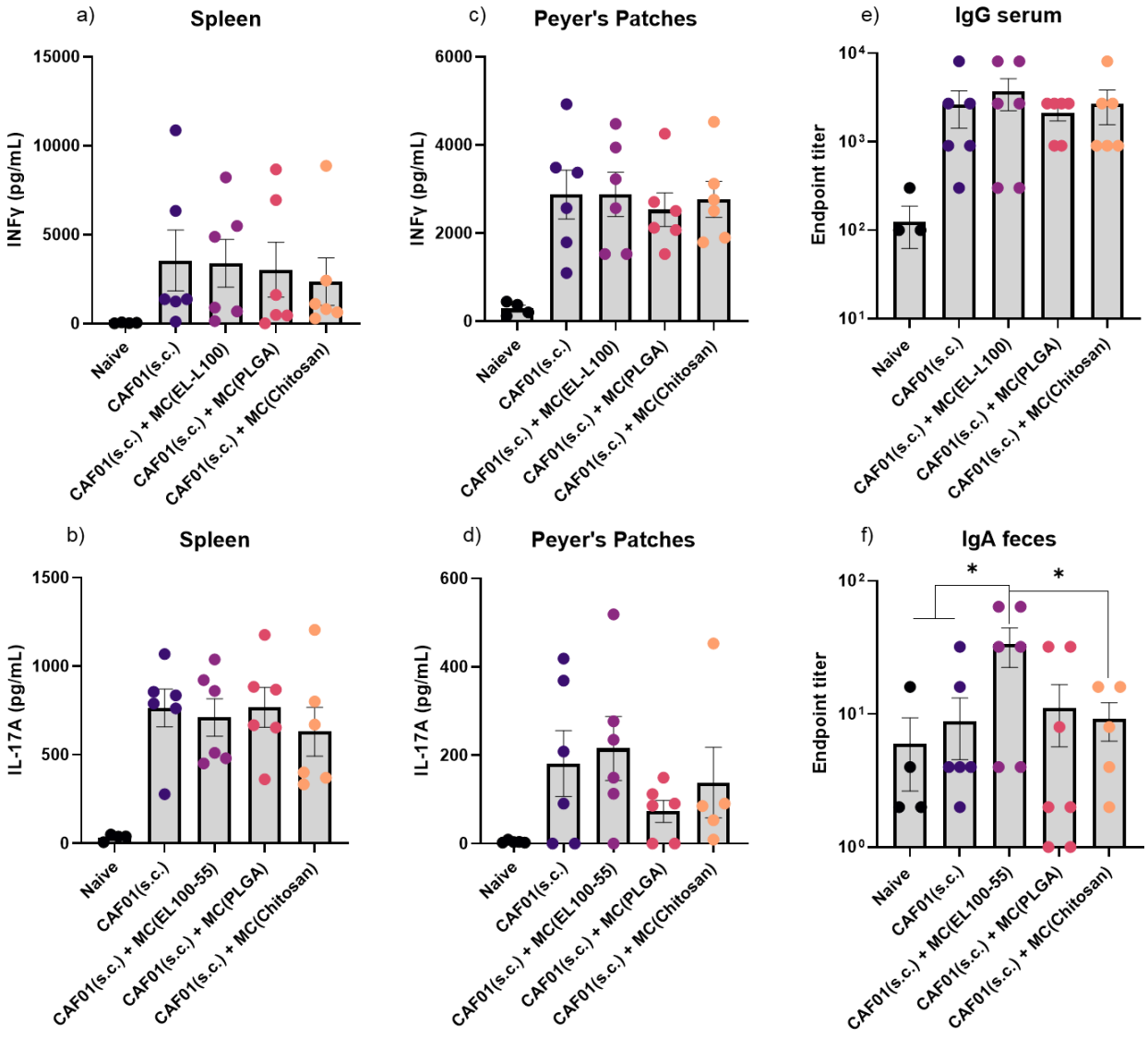

Fig. 4. Levels of secreted IFN-γ and IL-17A measured in spleen (a, b) and Peyer's Patches (c, d). CTH522 specific serum IgG and fecal IgA antibody titers (e, f). Mice, except the naive and CAF01(s.c.) group, were immunized 3 times in a prime-booster-booster regime, with either a sub cutaneous (s.c.) prime injection followed by oral boosters with

gavage or oral prime followed by oral boosters with gavage. Immunizations were given at day 0, 21 and 42. Data are shown from individual mice and bars represent mean ± SEM (n = 6). * $p < 0.05$.

The reason for this was further investigated, by visually tracking the release of the vaccine formulation from the MCs. Chips with MCs were coated with EL100-55 (Fig. 5a), PLGA (supporting information) or chitosan (supporting information) subsequent to vaccine loading, and emerged in maleic acid of pH 4.7 and 6.6 at 36.6 ^{o}C simulating stomach and intestinal conditions of mice, in the same manner as previously presented [24]. After 60 min in pH 4.7, lids of EL100-55 (Fig. 5b) and PLGA were still intact, however the majority of the chitosan lids had disappeared along with the content of the containers. A property of chitosan is a swelling behavioral trait, which can be utilized to achieve a slow sustained release if correctly engineered [38], [39]. The coating formulation used in this experiment does however, not seem to be compatible with the MCs and has probably detached itself upon swelling. This is most likely the reason why no effect was seen in the immunological analysis, due to an early release of the vaccine formulation in the stomach, rendering it ineffective. After 60 min, the MC chips were moved to pH 6.6 for 2x30 min. It is evident that the EL100-55 gradually disappears along with the content of the MCs (Fig. 5c, 5d). PLGA does still appear to be intact at these conditions, indicating that no release of vaccine occurs, making it the probable cause to why no effect was observed in the immunological analysis. PLGA has numerous times been employed as a particular delivery vehicle, and does seem to have adjuvant effects when used in this format, due to the particle morphology [34], [40]. As a coating however, this trait is obviously not exploited, and the PLGA formulation used in this study does not seem very well suited for the purpose of proximal intestinal release. However, it has been reported that PLGA can be tailored for colon-directed release, by modifying the lactic and glycolic ratios in combination with pH degradable polymers [41]. A PLGA formulation optimized for the purposes of this study could then most likely be manufactured. From another perspective, a colon targeting PLGA formulation, could be useful for vaccine-related purposes to stimulate local responses against colonic infecting pathogens. In this study, the immunological analysis and subsequent troubleshooting of the release of CTH522 formulation from MCs, revealed EL100-55 as the most proficient coating, for the purpose of oral vaccine delivery with MCs. A significant higher IgA response was observed in fecal samples from this group, most likely due to intended release of CTH522 in the intestine.

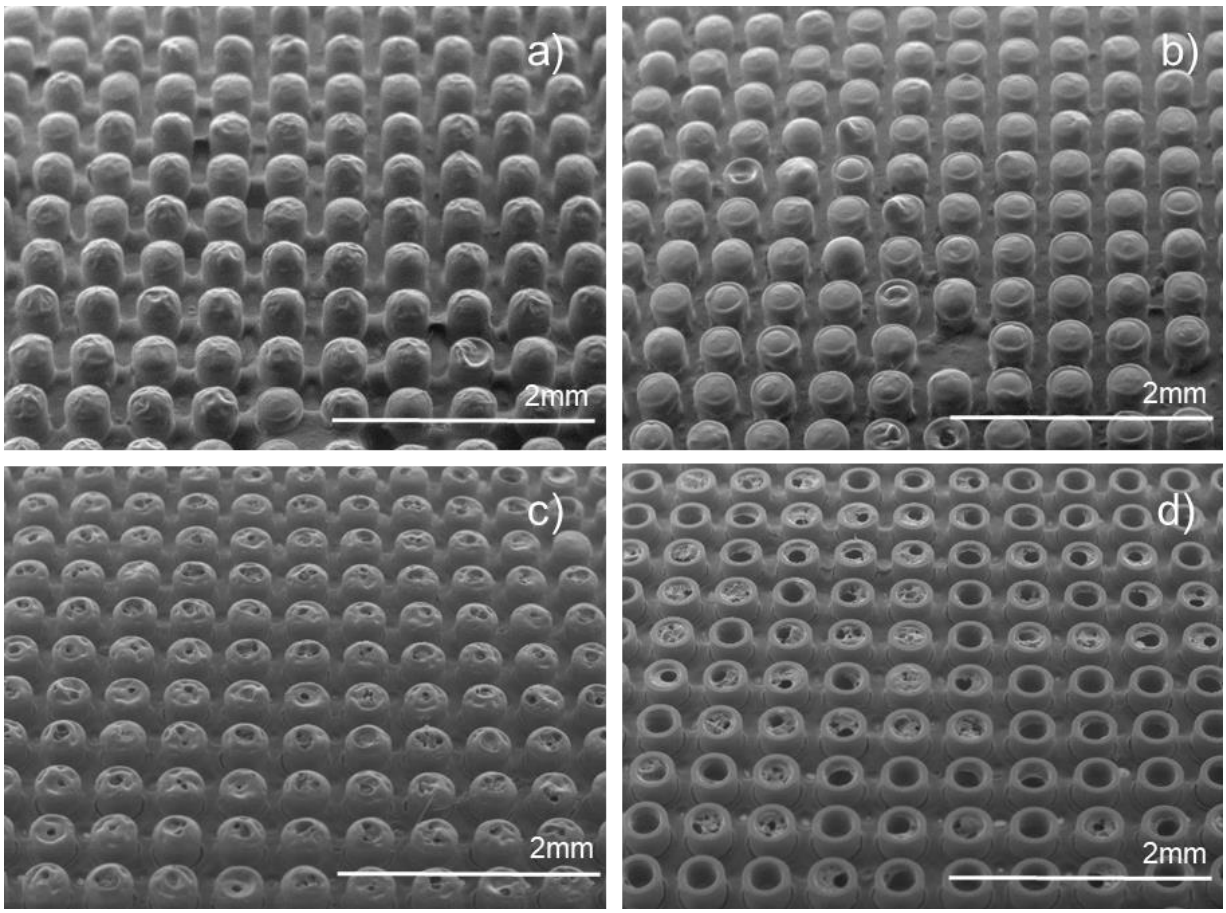

Fig. 5. SEM images showing dry microcontainers (MCs) on a chip loaded with CTH522 + α-GalCer coated with EL100-55 (a). MCs were soaked in 36.6˚C 2mM maleic acid at pH 4.7 simulating the environment of the mouse stomach and imaged after 60 min (b). MCs were then transferred to 36.6˚C 10mM maleic acid at pH 6.6 simulating the environment of the mouse intestine and imaged after 30 min (c) and 60 min (d).

3.4 Immunological analysis of oral delivery of CTH552 + α-GalCer with microcontainers coated with EL100-55.

Based on the results obtained from the screening of adjuvants and MC coatings, CTH522 was formulated with α-GalCer and dosed in MCs with EL100-55 lids, both as oral prime and boosters and as oral boosters following an s.c. prime with CTH522 + CAF01 (Fig. 6). Increased systemic IL-17 levels were observed in the groups receiving oral boosters with either MCs or gavage, compared to the group only receiving a s.c. prime indicating that the response is stimulated by the oral boosters, although not significantly enhanced (Fig. 6b). Furthermore, the IL-17 cytokine levels were slightly higher in the group boosted with MCs compared to the gavage group. The local immune response in PPs and IgA levels in feces, also showed a trend towards enhanced levels in the MC group compared to the s.c. and oral gavage groups, but not significantly higher (Fig. 6c, 6d, 6f). *Davitt et al.* demonstrated a significantly enhanced systemic and local response of cholera specific IgA antibodies, along with increased INFy levels in the PPs, from dosing Dukoral adjuvanted with α-GalCer orally [42]. These findings are in line with the trends observed in the local mucosal responses in the present study, however the stimulated responses were not statistically enhanced. A likely cause for the different findings is that Dukoral contains a killed whole-cell antigen, thus benefitting from intrinsic adjuvant traits as opposed to CTH522 [43], [44]. An interesting observation from this study is the stimulation of Th17-cells in PPs, which is not seen in the study by *Davitt et al*. CAF01 is known to induce systemic Th17 responses, that can be pulled into mucosal tissues after local mucosal

vaccination. The Th17 responses observed in this study could thus be an example of this prime-pull effect, where the Th17 cells, generated by the s.c. prime injection, are migrating to the intestinal tissue upon oral boosting [10], [45], [46]. Elevated levels of Th1 and Th17 responses in PPs as well as IgA antibody titers were also observed in the oral MC prime-boost group compared to the naive mice, indicating that the CTH522 + α-GalCer formulation is indeed capable of inducing mucosal immune responses when delivered orally in MCs ($p = 0.0436$, 0.0273, $p = 0.0281$, Fig. 6c, 6d, 6f). Albeit the oral MC prime-boost group did not reach the same level of immune induction as the prime-pull groups.

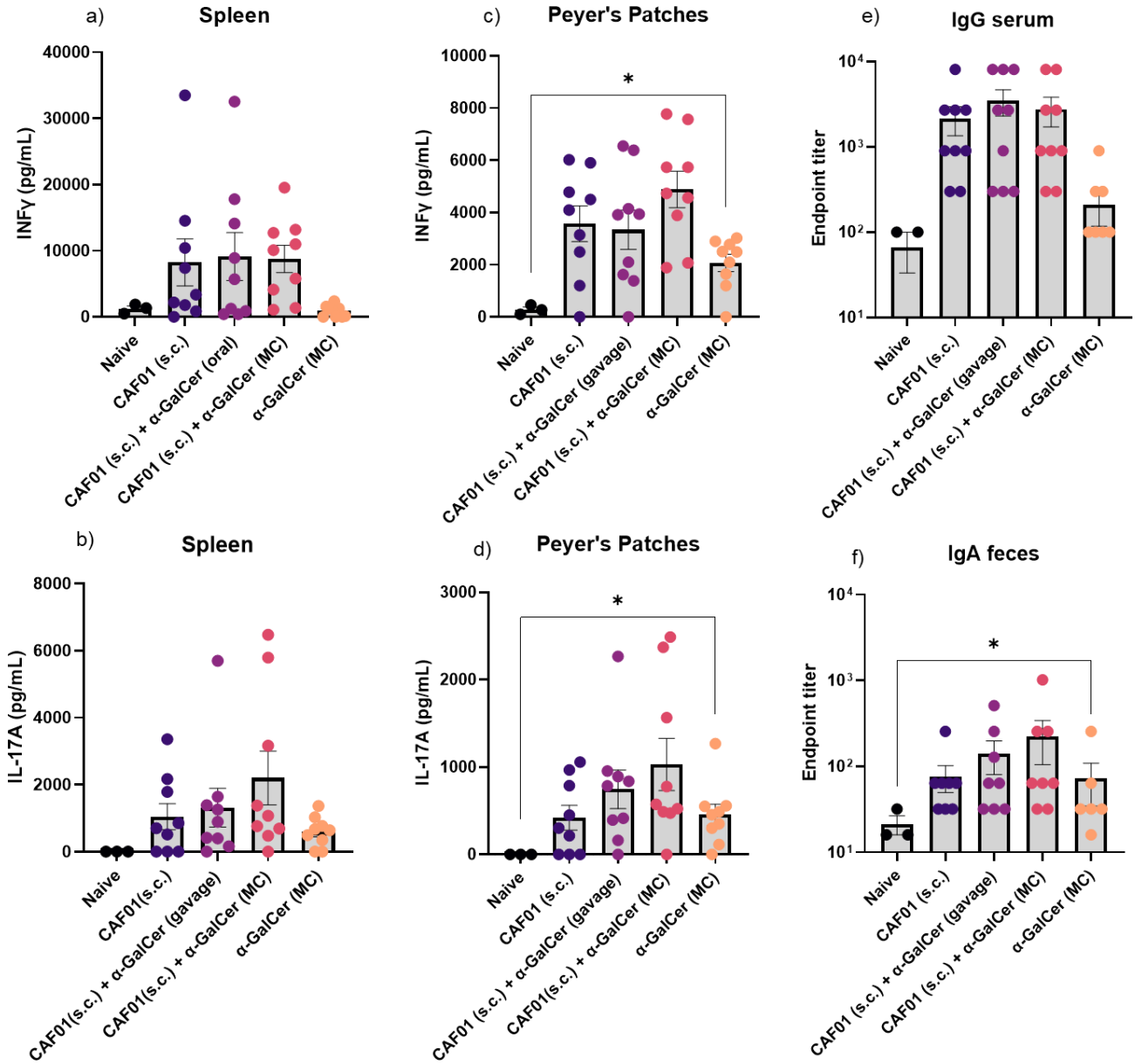

Fig. 6. Levels of secreted IFN-γ and IL-17A measured in spleen (a, b) and Peyer's Patches (c, d). CTH522 specific serum IgG and fecal IgA antibody titers (e, f). Mice, except the naive and CAF01 (s.c.) group, were immunized 3 times in a prime-booster-booster regime, with either a subcutaneous (s.c.) prime injection followed by oral boosters with gavage or microcontainers (MCs). One group received oral prime followed by oral boosters with MCs. Immunizations were given at day 0, 21 and 42. Data are shown from individual mice and bars represent mean ± SEM (n = 9). * $p < 0.05$.

3.5 CT-scan and X-ray imaging of microcontainer transit kinetics in mice

Even though we observed measurable immune responses upon delivery of adjuvanted CTH522 in MCs, levels were lower than expected. It was therefore speculated that the MCs transit is either too fast or that they deliver the vaccine to the wrong intestinal compartment for stimulation of the immune cells to occur. A recent study by *Esterházy et. al.* thus demonstrated that draining lymph nodes in the distal intestine, promoted effector T-helper cells, whereas proximal lymph nodes promoted T_{reg} responses [47]. These findings indicate that release of vaccine in the proximal intestine may not be optimal, whereas the distal intestinal compartment could be highly relevant to target [1], [47].

X-ray and CT-scanning have before been used to thoroughly investigate transition in rats but never in mice [25]. Combined analysis of these methods showed that the transit time from the stomach to the cecum of MCs in mice was about 1-1.5 h (Fig. 7). This corresponds to the standard transit time of the GI tract in mice, and could be the reason why a higher immune response was not achieved [48]. It is therefore possible that the immunological performance could be enhanced, by tailoring the polymeric lid to release in the distal part of the intestine. The MCs are made of the mucoadhesive material SU-8 and have previously been proved to increase retention of MCs in the intestine of rats [25], [49]. This effect was thus expected to be utilized in mice as well for the purpose of oral vaccine delivery. However, the data from rats taken together with the observed results in this study, indicate that the proportional size of the MCs to mice is too large, and the MCs are effected by peristaltic movements and moved regardless of being mucoadhesive. A solution could be to change the animal model to one proportionally larger and with a longer transit time, such as rabbits or pigs. This would additionally improve the scalability, and give vital information on the MCs kinetics in an environment genetically and metabolically closer to that of humans[50]. Ideally, a process should be put in place, designed to first get indication of promising formulation candidates in smaller animal models, which then should continue to testing with the microdevices in larger animals. Methods to employ to get indications of the formulations potential, could be intra-intestinal infusion, in combination with an intestinal closed-loop model, where the infused material is prohibited from transit through the intestine [51], [52]. This would in theory evaluate the MCs ability to deliver the vaccine to immune cells of the distal part of the intestine, should it be retained long enough in addition to vaccine formulations immunological capabilities when properly delivered. To further optimize the MC technology for oral delivery of vaccines, the retention time in the intestine should most likely be prolonged. Furthermore, the targeting of immunogenic sites, such as the M-cells in PPs, could greatly benefit the technology to enhance interaction with the immune-cells and the achieved response.

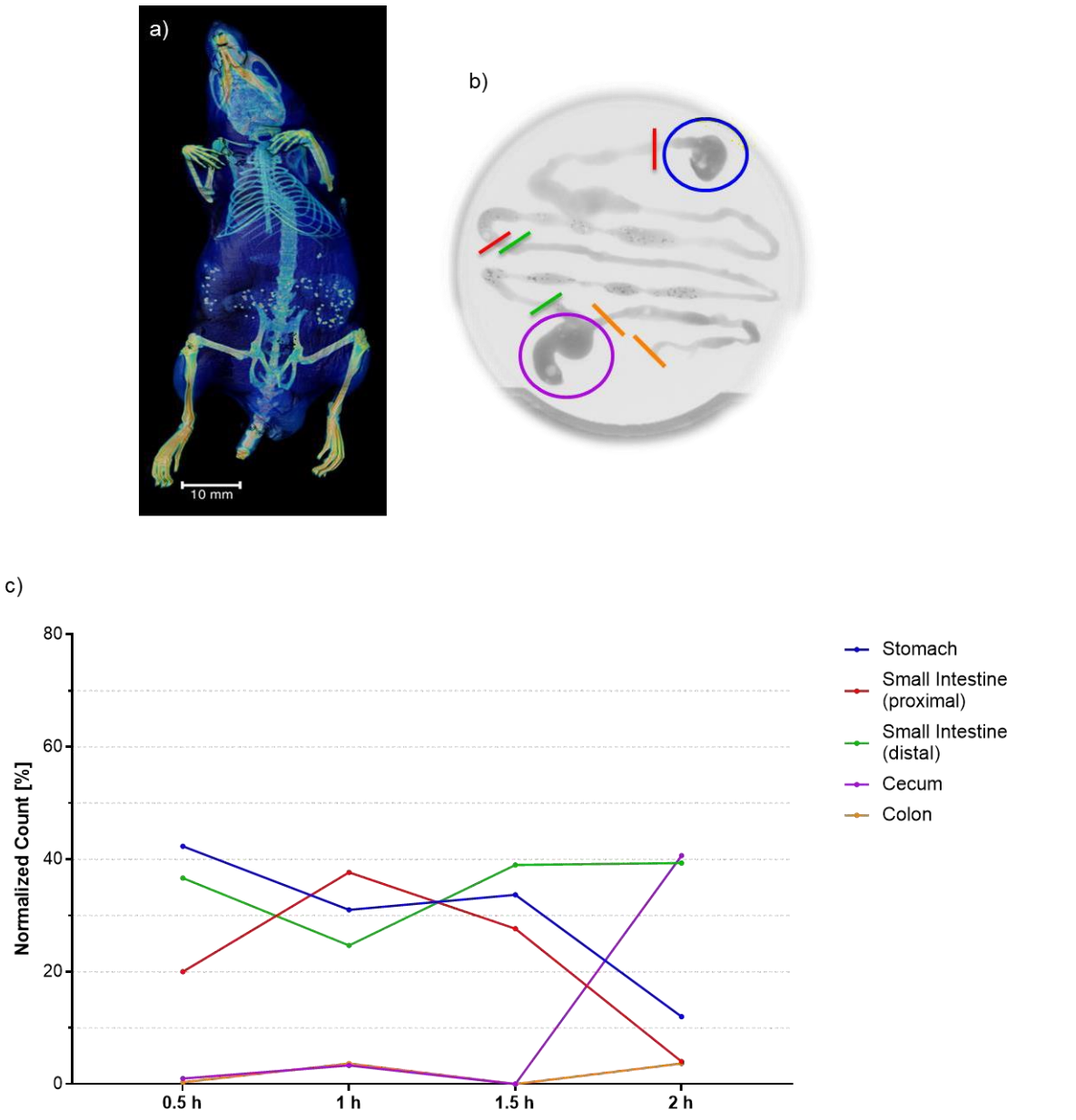

Fig. 7. CT-scan of mouse 2 h post-administration with $BaSO_4$ loaded microcontainers (MCs). X-ray of isolated GI tract of mouse 1.5 h post-administration with $BaSO_4$ loaded MCs with indication of stomach (blue), proximal small intestine (red), distal small intestine (green), cecum (purple) and colon (orange) (b). Graph showing amount of BaSO4-loaded MCs found in each section of the isolated GI tracts for each time point of euthanasia (c). The counts were found combining CT-scan and planar X-ray imaging and are here plotted as single points, with lines linking each point (n = 3).

4. Conclusion

In this work, we tested MCs ability to orally deliver the *C. trachomatis* vaccine candidate CTH522 in combination with a mucosal adjuvant. α-GalCer was found to be the most prominent adjuvant to be formulated with CTH522. A procedure to successfully lyophilize the vaccine formulation, without degrading the antigen or losing immunogenicity, was developed. MCs coated with EL100-55 elicited a significantly higher local CTH522 specific IgA response, compared to MCs coated with PLGA and chitosan, deeming EL100-55 the best choice for MC lids. CTH522 + α-GalCer administered in MCs orally following an s.c. prime, showed an increase in the mucosal immune response locally and to a degree systemically, demonstrating a prime-pull effect. Solely oral dosing with MCs also managed to generate significantly enhanced mucosal immune responses compared to naive mice. Some optimization is however needed, as the measured immune responses are relatively low, and not significantly enhanced compared to just receiving an s.c. prime. A possible reason for this could be the fast transit time in mice. CT-scan and X-ray imaging showed that the transit time of MCs delivered orally is only 1-1.5h and that they are not retained despite mucoadhesive traits. This is probably partly due to the proportional size ratio between the MCs and the intestinal tract of mice. In a mouse, the diameter of the intestinal tract is approximate 2mm, and the mucus layer is around 20-25 µm thick. Thus, the microcontainers will not be completely embedded in the mucus layer and will then easily be moved along with consumed food and peristaltic movements. Our results indicate that the mouse is not an optimal animal model, when dealing with oral delivery of devices in the 100 µm range. In future studies with such devices, larger animals may be needed to study the effects of MCs. Assuming the MC technology was implemented as a vaccination solution for humans, the results in this paper suggest that people would need a prime injection, followed by oral MC boosters. The ideal situation would be, that patients could administer capsule themselves making the procedure more compliant and time effective. However, in order to account for the potential humane errors, such as unproper storage of capsules, failing to administer them, etc. it would be more feasible for patients to appear at vaccination centers for administration. In this case it would most likely still be more time efficient than the mass vaccination under COVID-19, with minimal need for medical personal. There is of course a lot of aspects in this, and several methods on how to ensure proper consumption and storage of the capsules by the patients could be discussed.

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Conflicts of interest

The authors have no conflict of interest to declare

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