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Sublingual Boosting with a Novel Mucoadhesive Thermogelling Hydrogel Following Parenteral CAF01 Priming as a Strategy Against *Chlamydia trachomatis*

*Lorena Garcia-del Rio, Patricia Diaz-Rodriguez, Gabriel Kristian Pedersen, Dennis Christensen, and Mariana Landin**

Chlamydia trachomatis **is the most prevalent sexually transmitted disease of bacterial origin. The high number of asymptomatic cases makes it difficult to stop the transmission, requiring vaccine development. Herein, a strategy is proposed to obtain local genital tract immunity against *C. trachomatis* through parenteral prime and sublingual boost. Subcutaneous administration of chlamydia CTH522 subunit vaccine loaded in the adjuvant CAF01 is combined with sublingual administration of CTH522 loaded in a novel thermosensitive and mucoadhesive hydrogel. Briefly, a ternary optimized hydrogel (OGEL) with desirable biological and physicochemical properties is obtained using artificial intelligence techniques. This formulation exhibits a high gel strength and a strong mucoadhesive, adhesive and cohesive nature. The thermosensitive properties of the hydrogel facilitate application under the tongue. Meanwhile the fast gelation at body temperature together with rapid antigen release should avoid CTH522 leakage by swallowing and increase the contact with sublingual tissue, thus promoting absorption. In vivo studies demonstrate that parenteral-sublingual prime-boost immunization, using CAF01 and OGEL as CTH522 vaccine carriers, shows a tendency to increase cellular (Th1/Th17) immune responses when compared to mucosal or parenteral vaccination alone. Furthermore, parenteral prime with CAF01/CTH522 followed by sublingual boosting with OGEL/CTH522 elicits a local IgA response in the genital tract.**

1. Introduction

Sexual transmitted infections lead to significant morbidity and mortality worldwide. Their high incidence rate has a great impact on healthcare systems, causing high social and economic costs.[1] *Chlamydia trachomatis* (Ct) is an intracellular pathogen that infects around 131 million people annually and can affect vaginal, pulmonary, and conjunctiva mucosae.[2,3]

Mucosal surfaces are the main entry site for most pathogens—including Ct—and are therefore protected by a highly specialized immune system. The stimulation of the mucosal immune system by pathogen invasion or vaccination can induce both systemic and local mucosal immune responses.[4,5] Furthermore, mucosal immunization can also elicit immune responses at remote mucosal surfaces due to the migration of activated B and T cells.[4,6,7] Therefore, sublingual and intranasal vaccination could confer protection against genital tract infections. Moreover, unlike vaginal immunization, sublingual and intranasal vaccination does not depend on the host hormonal status.[8]

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Nasal mucosa has been used as an effective immunization route against Chlamydia and other sexually transmitted infections.[3,9,10] However, this strategy presents safety concerns since the administration of certain enterotoxins used as adjuvants by nasal route has been associated with Bell's palsy in some patients.[6,8,11] On the contrary, recent clinical trials have shown that sublingual vaccination allows to decrease the infectious episodes of the urinary tract with only rare side effects and no facial nerve disturbance after enterotoxin administration.[12,13] Furthermore, the results of sublingual administration of the quadrivalent human papillomavirus vaccine Gardasil® show this route can be used for boosting and inducing functional antibody responses.[14] Finally, studies in mice immunized against Chlamydia have shown a stronger immune response when parenteral and mucosal vaccines were given in tandem.[15,16]

The sublingual route is accessible for drug administration and patient-friendly. Moreover, sublingual tissue contains plenty of antigen-presenting dendritic cells that can recirculate to distant lymph nodes and spleen, and also induce IgA and IgG antibodies and cytotoxic T cells in the genital tract.[17,18] However, sublingual vaccine administration in both humans and non-human primates is challenging and may fail to elicit mucosal immunity.[12,14] These discouraging results are most probably caused by poor antigen delivery due to swallowing of vaccine components. Jones et al. thus demonstrated in macaques that the administration of HIV-1 antigens directly in the oral underlying tissue using a needle-free injector, ensuring reduced loss due to swallowing, facilitates increased uptake by dendritic cells, and the development of strong humoral and cellular responses in both systemic and mucosal compartments.[19] Thereby, the development of delivery systems able to protect the antigen(s) from salivary enzyme degradation and promote antigen permeation through sublingual mucosa might be the answer to achieving better mucosal immune responses.

A promising strategy could be the development of mucoadhesive thermogelling hydrogels. These formulations can be easily administered under the tongue due to their liquid nature and resist wash out by becoming semisolid at body temperature.[20] Thus, antigens can remain in contact with sublingual mucosa for longer, facilitating increased uptake by dendritic cells. In addition to acting as carriers for antigens, hydrogels may have intrinsic immunostimulatory properties, and can also be loaded with other adjuvants, such as dmLT and CpG.[21,22]

As described above, the combination of systemic and mucosal routes is beneficial to induce more robust immune responses against Chlamydia.[15,16,23] Herein we propose a systemic priming followed by a sublingual boosting as the vaccination strategy for Ct. For that purpose, Chlamydia subunit vaccine CTH522, which is a recombinant Ct outer membrane protein comprising immunorepeats of the D, E, F, and G Ct serovars, was used.[24] CAF01 (cationic adjuvant liposomes type 01) is an adjuvant composed of dimethyldioctadecylammonium bromide (DDA) and glycolipid trehalose 6,6´-dihibenate (TDB), successfully used in several clinical trials, including one trial with CTH522, demonstrating its safety and tolerability.[24–27] This adjuvant has furthermore been shown to prime a parenteral immune response that supports the establishment of mucosal memory upon mucosal boosting.[28,29] Parenteral administration of CAF01-adjuvanted CTH522 combined with intranasal boosting using the same formulation induced a synergistic protective effect against Chlamydia in minipigs.[23] However, systemic priming using CAF01 followed by sublingual boosting has not been explored. In order to maintain the contact of CTH522 with the sublingual mucosa and favor its permeation and uptake by dendritic cells, a novel and optimized mucoadhesive hydrogel (OGEL) was proposed.[20] The optimal ternary composition was selected using artificial intelligence techniques giving rise to a hydrogel with desirable biological and physicochemical properties.

The aim of the present work was to explore the usefulness of sublingual boosting with vaccine-loaded mucoadhesive thermosensitive hydrogels posterior to systemic priming as a strategy for immunization against Chlamydia. For this purpose, the Chlamydia CTH522 vaccine antigen was administered in two sequential doses. First subcutaneously using CAF01 as adjuvant and then sublingually using either CTH522 alone or loaded into hydrogels. The capacity to induce immune responses of two sequential sublingual administrations with the CTH522-loaded hydrogel was also evaluated.

2. Experimental Section

2.1. Materials

Pluronic F127 (PF127) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (USA). Hybrane S1200 (HS1200) was acquired from Polymer Factory (Sweden). Gantrez AN119 (AN119) was kindly provided by Ashland (Spain). Trehalose 6,6´dibehenate (TDB) and dimethyldioctadecylammonium bromide (DDAB) were purchased from Avanti Polar Lipids Inc. (USA). Cell Strainers presenting a pore size of 40 µm and 100 µm were purchased from Corning Incorporated (USA). Purified rat anti-mouse IL-17A, biotin rat anti-mouse IL-17A, purified rat anti-mouse IFN-γ, biotin rat anti-mouse IFN-γ and streptavidin HRP were purchased from BD Pharmingen (USA). Rabbit anti-mouse IgG (H+L)-HRP was obtained from Invitrogen (USA). Goat anti-mouse IgA-HRP was purchased from Southern Biotech (USA). Carbonate buffer pH 9.6 was supplied by SSI Diagnostics (Denmark). TMB PLUS2 was obtained from Kem-En-Tec Diagnostics (Denmark). *Chlamydia trachomatis* vaccine CTH522 was supplied by Statens Serum Institut (SSI) (Denmark).

2.2. Hydrogel Development and Preparation

The database generated in our previous investigation (Table S1, Supporting Information)[20] was used to create a model with the commercial software INForm v.5.01 (Intelligensys, Ltd. UK) which combines artificial neural networks (ANN) and genetic algorithms. The database includes 19 records of ternary hydrogel formulations of different compositions. The independent variables were, Gantrez variety (AN119 or S97), and the percentages of Gantrez, Pluronic F127, and Hybrane S1200. Additionally, the dependent variables were the rheological (gelation temperature), texturometric (mucoadhesion and adhesion work, cohesion and gel strength at 37 °C), and release properties of the produced hydrogels.

Table 1. Training parameters used for INForm modeling.

ANN network structure:	**Test data:**
No. of hidden layers = 1	Screen update set = 5
No. of nodes in hidden layer = 2	Smart stop enabled
Transfer functions:	Minimum interactions = 20
Hidden layer transfer function = Asymmetric sigmoid	Test error weighting = 0.2
Output transfer function type = Linear	**Optimization**:
Back propagation type = RPROP	Number of populations = 1
ANN targets:	Number of iterations = 100
Targets epochs = 1000	Population size = 100
Target MS error = 0.0001	Replacement % = 50
Random seed = 10 000	Mutation standard deviation = 0.1
	Random seed = 1

Data were split into two sets, 16 records for training and 3 for error testing. The parameters selected for INForm modeling are shown in **Table 1**.

The accuracy of the ANN models was assessed using the determination coefficient (R^2) (Equation 1), calculated for train and test data:

$$R^2 = \left[1 - \frac{\sum_{i=1}^{n}\left(y_i - y_i^*\right)^2}{\sum_{i=1}^{n}\left(y_i - y_i^{**}\right)^2}\right] \times 100 \quad (1)$$

where, y_i represents the output's experimental value in the data set, y_i^* is the predicted value of the output predicted by the ANN model and finally, y_i^{**} is the mean value of the parameter that is being evaluated.

The optimal hydrogel formulation (OGEL) obtained following the INForm model was produced following the protocol previously reported by Garcia-del Rio et al.[20] Briefly, an accurate amount of Gantrez (AN119) was dissolved in Milli-Q water at 50 °C. Then, Hybrane S1200 (HS1200) was incorporated into this solution, previously cooled in an ice bath. Finally, Pluronic F127 (PF127) was added and dissolved overnight at 4 °C, and pH was adjusted to 6.8. Afterwards, hydrogel sterilization was carried out by autoclaving (Trade Raypa steam sterilizer AES-12, Barcelona, Spain). Sterile hydrogels were stored at 4 °C until use.

2.3. Hydrogel Characterization

2.3.1. Rheological Properties

A rheometer AR1000-N (TA Instruments, UK) was used to estimate hydrogels' storage (G′) and loss moduli (G″), using a cone-plate geometry of 6 cm Ø and 2.1 degrees. Both moduli were recorded from 15 to 40 °C at 0.4 °C min^{-1} at an angular frequency of 5 rad s^{-1}. Gelation temperature (T_{gel}) was established by the crossover of both moduli.

2.3.2. Texturometric Properties

A TA XT plus Texture Analyzer (Surrey, UK) was used to obtain the texturometric profile of the optimized hydrogels. All texturometric properties (mucoadhesion, gel strength, adhesion, and cohesion) evaluated in this work were performed at 37 °C following protocols previously described.[20]

Briefly, bovine buccal tissue was used as a model of oral mucosa to measure mucoadhesion work. Buccal mucosa was cut into pieces of 1.5 cm x 1.5 cm and glued to the punch of the texturometer, then a Petri dish containing 3 g of OGEL was attached to the lower support of the texturometer. The punch descended at 1 mm s^{-1} and applied a force of 0.5 N on the optimized hydrogel for 60 s. The mucoadhesion work (mJ) was calculated as the force exerted by the punch when rising at 1 mm s^{-1}.

To estimate gel strength, adhesion work, and cohesion, two compression cycles were performed. As for mucoadhesion, the punch descended at 1 mm s^{-1} and compressed the hydrogel to 5 mm in depth. After a required relaxation time of 30 s, a second compression cycle was carried out. Gel strength (N) and adhesion work (mJ) were calculated using the force-distance plot generated during the first compression cycle. Meanwhile, for cohesion assessment both force-distance plots were required.[20]

2.3.3. Protein Release

Bovine serum albumin (BSA), having a similar molecular weight to CTH522,[30] was used as a protein model to assess OGEL release properties following the protocol established by Garcia del Rio et al.[31] with slight modifications. BSA was dissolved in OGEL reaching a final concentration of 0.75 mg ml^{-1} and stored at 4 °C overnight. Release studies were performed at 37 °C and 400 rpm in an orbital shaker Tritamax 1000 (Heidolph Instruments, Germany) using simulated salivary fluid (SSF) as release media.[32] Then, 2 ml of the loaded hydrogel were placed in a Corning Cell Strainer of 40 µm pore size, previously located in 6-well plates containing 8 ml of SSF. The amount of BSA released was quantified employing the Pierce BCA Protein Assay according to the manufacturer's protocol. Absorbance was measured using a FLUOstar OPTIMA plate reader (BMG Labtech, Germany) and the percentage of BSA released at 5 min was calculated using adequate controls and validated calibration curves.

2.3.4. Stability Properties

The effect of storage on the properties of OGEL was evaluated after one year at 4 °C in closed containers. Texturometric, rheological, and release properties were re-analyzed after storage.

2.4. CAF01 Liposomes Preparation

CAF01 liposomes were obtained through the hydration method, allowing the formation of large and heterogeneous multilamellar vesicles as it was previously reported.[33] Briefly, both lipids, TDB and DDA, were individually dissolved in a mixture of chloroform and methanol (9:1 v/v). Afterwards, accurate amounts of the lipid

solutions were transferred to round-bottom flasks to get a final concentration of 1 mg ml^{-1} for TDB and 5 mg ml^{-1} for DDA, and rota- evaporated for 15 min at 200 rpm to remove the organic solvent. Finally, the desired volume of 10 mM Tris buffer (pH 7.4) was added and the lipid film was hydrated for 20–30 min at 60 °C.

2.5. Antigen and Formulations Loading

CAF01 and OGEL formulations were loaded under sterile conditions with the antigen selected, the recombinant CTH522 chlamydia protein.[34]

CAF01 liposomes were loaded by dissolving CTH522 in Tris-buffer (10 mM, pH 7.4) supplemented with 9% trehalose and then mixed in a 1:1 ratio with CAF01. The final CTH522 concentration was 0.025 μg μl^{-1}.

An accurate amount of CTH522 stock solution was added to OGEL hydrogel to achieve an antigen concentration of 0.2 μgμl^{-1}, and gently mixed in a vortex. Loaded OGEL was stored overnight at 4 °C to ensure total bubbles removal.

2.6. In Vivo Assays

2.6.1. Ethics Statement

Animal experiments were conducted at Statens Serum Institut (SSI) according to the regulations established by the Danish Ministry of Justice and animal protection committees by the Danish Animal Experiments Inspectorate Permit 2017-15-0201-01363 and in compliance with the European Community Directive 2010/63. All protocols have been subjected to ethical review and approved by SSI.

2.6.2. Mouse Immunization

Seven to nine-week old CB6F1 female mice were housed at SSI and handled by authorized staff. Animals were divided into four groups. Groups 1 and 4 consisted of three animals, while groups 2 and 3 consisted of four and five animals, respectively. The immunization schedule is depicted in **Figure 1**. Mice immunized sublingually were anesthetized with a Zoletil mixture.

Groups 1 to 3 were subcutaneously primed at the base of the tail with 200 μl of CAF01/CTH522, which contained 5 μg of CTH522, 250 μg of DDA, and 50 μg of TDB. Group four was sublingually primed with 10 μl of OGEL (OGEL/CTH522), which contained 2 μg of CTH522 dispersed in the hydrogel. After mucosal vaccination, mice were placed in ante-flexion posture for 20 min. Then, groups 2 to 4 were boosted twice sublingually (days 15 and 35), with 2 μg of CTH522 alone (Group 2) or included within 10 μl of OGEL system (Groups 3 and 4). Group 1, vaccinated only with CAF01/CTH522 was established as control. Mice were euthanized 3 weeks after the last mucosal boosting.

2.6.3. Antibody Titers in Blood and Vaginal Samples

At the end of the study, an enzyme-linked immunosorbent assay (ELISA) was employed for the quantification of IgA and total IgG in serum and vaginal lavage. Briefly, blood samples were collected from the tail vein in a heparinized tube (1% w/v heparin) and centrifuged at 10 000 *g* for 10 min to separate sera from cells. Serum samples were stored in a 96-well plate at −20 °C until use. Vaginal wash samples were obtained by washing out the vagina with 100 μl of phosphate-buffered saline (PBS) and stored at −80 °C until further use. Vaginal samples were treated with bromelain (2.5%) and diluted 5 times with phosphate-buffered saline containing 1% of BSA similarly to what has already been reported.[34–36] Afterwards, samples were incubated for 1 h at 37 °C to break the vaginal mucous network. 96 well MaxiSorp flat bottom plates were coated overnight at 4 °C with 100 μl per well CTH522 (1 μg ml^{-1}) diluted in carbonate buffer (pH 9.6), and then blocked with 200 μl per well PBS (pH 7.2) containing 2% BSA during at least 1.5 h at room temperature. Then vaginal (37.5 μl) or serum (12.5 μl) samples were added in serial dilutions and incubated for 2 h at room temperature. Finally, antigen-specific IgA and total IgG were detected employing HRP-conjugated goat anti-mouse IgA or rabbit anti-mouse IgG antibodies respectively, TMB substrate and sulfuric acid (0.2 M). Plates were measured at 450 nm with wavelength correction (570/620 nm) in a spectrophotometer (Sunrise Tecan, Austria).

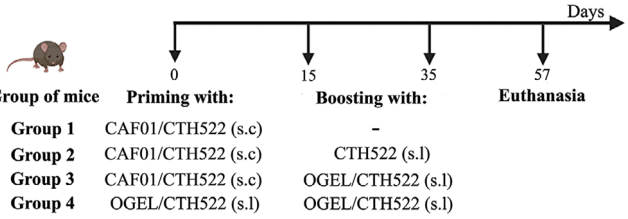

Figure 1. Immunization schedule and experimental groups tested. Animals were primed subcutaneously (s.c) or sublingually (s.l) using CAF01 and OGEL as vaccine delivery systems, respectively. Thus, mice from Groups 1, 2, and 3 were immunized with CAF01/CTH522 (s.c) meanwhile Group 4 was mucosally vaccinated with OGEL/CTH522 (s.l). On days 15 and 35, except animals from Group 1, mice were boosted twice through sublingual (s.l) route with CHT522 alone (Group 2) or loaded into OGEL formulation (Groups 3 and 4).

2.6.4. IFN-γ and IL-17A Responses in Spleen and Cervical Lymph Nodes

IFN-γ and IL-17A produced by splenocytes and cervical lymph nodes were evaluated through a sandwich ELISA. Individually in 6-well cell culture plates, organs were disrupted with cell strainers of 100 μm pore size using sterile syringe pistons. Each strainer was washed with PBS (pH 7.2) and cell suspensions were transferred to 15 ml tubes and filled with PBS (pH 7.2) supplemented with 10% foetal calf serum (FCS) up to 12 ml. Then, tubes were spun down at 2000 rpm for 5 min. Supernatants were discarded and cell-pellets were suspended in 12 ml of RPMI media. Samples were spun down again, supernatants were withdrawn, and cell-pellets suspended in either 0.5 or 2 ml of RPMI media for lymph nodes or splenocytes respectively, subsequently, the cell concentration of each sample was quantified using a Nucleo counter (Chemometec, Denmark). Afterwards, known amounts of cell suspensions were diluted in RPMI media supplemented with FCS (10%), HEPES buffer (1%), penicilin-streptomycin, L-Glutamine, sodium pyruvate, and non-essential

amino acids to get a concentration of 2×10^6 cells ml^{-1}. Then, 100 µl of each cell-suspension were transferred to 96-cell round bottom plates and re-stimulated with 100 µl of RPMI media, CTH522 (5 µg ml^{-1}) or Concanavalin A (3 µg ml^{-1}) for 72 h at 37 °C, 5% CO_2, and 95% humidity. Cells re-stimulated with media alone and Concanavalin A were used as negative and positive controls, respectively. Lastly, supernatants were collected and stored at −20 °C until use. Secreted IFN-γ and IL-17A cytokines in splenic and lymph nodes supernatants were quantified using ELISA kits following protocols reported elsewhere.[33] Briefly, specific amounts of purified rat anti-mouse IL-17A or IFN-γ were diluted in carbonate buffer (pH 9.6), added (100 µl per well) to MaxiSorp plates, and incubated overnight at 4 °C. Then, plates were blocked with PBS containing skimmed milk powder (2%) for 1.5–2 h at room temperature and, subsequently, plates were incubated for 2 h with 2% BSA in PBS. Finally, IL-17A or IFN-γ concentration was determined using standard curves and the corresponding biotin anti-mouse antibody, streptavidin, TMB substrate, and sulfuric acid (0.2 M). Plates were read at 450 nm with wavelength correction (570/620 nm).

2.7. Statistical Analysis

Results from hydrogel characterization and in vivo experiments are expressed as the mean and standard error of the mean (SEM). SPSS software (IBM SPSS Statistics 26) was employed to perform a one-way ANOVA and HSD Tukey's post hoc tests ($p < 0.05$).

3. Results

3.1. Hydrogel Preparation, Characterization and Stability

Combinations of PF127, HS1200, and AN119 polymers have been demonstrated to be useful for obtaining thermosensitive and mucoadhesive hydrogels.[20] INForm succeeded in modeling the composition variables as inputs and the rheological, texturometric, and release properties as outputs. Models for all the parameters had Train R^2 higher than 95% and test R^2 over 82%, indicating excellent predictabilities. The generated INForm model was asked about how to get hydrogels with gelation temperature between 28 and 32 °C together with maximum mucoadhesion, adhesion work, cohesion, and gel strength at 37 °C and fast release. The model predicted that optimal hydrogels can be obtained with the following composition: 21.10% of PF127, 5.00% of HS1200, and 0.08% of Gantrez AN119 (Total desirability = 77.5%).

The properties of the optimal hydrogel composition (OGEL) selected by INForm following Table 1 conditions, were close to those predicted, validating the obtained ANN model. As shown in **Table 2**, OGEL is a thermosensitive system that gels at 19.7 °C and thus immediately after application within the mouth. It has a gel strength of 0.7 N, a mucoadhesion and adhesion work of 1.24 and 1.74 mJ respectively, and a cohesion of 0.2. Finally, it releases up to 41.11% of the protein at 5 min, quick enough to ensure vaccine absorption in a few minutes[37–39] and avoids formulation leakage by swallowing. OGEL has long-term stability at 4 °C (Table 2) and statistically significant differences could not be found between OGEL freshly prepared and after 1-year storage at 4 °C ($p < 0.05$).

Table 2. Values predicted by the ANN model and experimental values for the parameters characterized for OGEL freshly prepared and after 1 year of storage at 4 °C in closed containers.

Parameter	Predicted by ANN	OGEL ($t = 0$)	OGEL ($t = 1$ year)
T_{gel} (°C)	20.6	19.7 ± 0.14	19.5 ± 0.23
Gel strength (N)	0.6	0.70 ± 0.01	0.67 ± 0.01
Mucoadhesion work (mJ)	1	1.24 ± 0.18	1.26 ± 0.15
Adhesion work (mJ)	1.5	1.74 ± 0.12	1.75 ± 0.05
Cohesion	0.3	0.20 ± 0.07	0.16 ± 0.00
% BSA released at 5 min	43.5	41.11 ± 1.88	44.2 ± 4.70

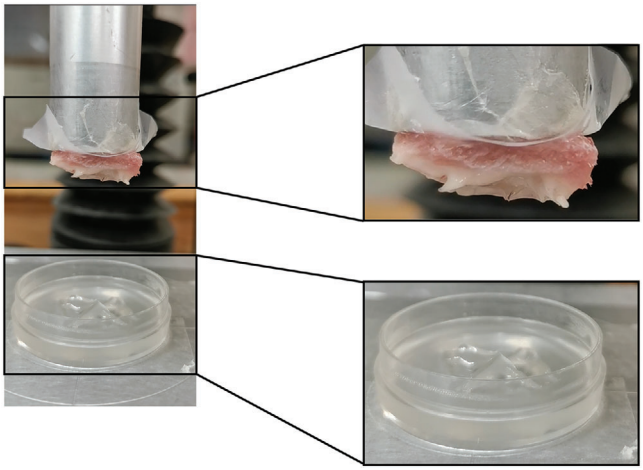

Figure 2. Image of OGEL after the contact with the bovine buccal tissue using the settings for the texturometric properties assessment.

The mucoadhesive character of OGEL is shown in **Figure 2**. After the contact with the bovine buccal tissue, part of OGEL attached to the mucosa while the remaining gel showed the surface morphology of the tissue.

3.2. Prime Immunization Using CAF01 Followed by OGEL Mucosal Boosting Improves Mucosal Antibody Responses

A mouse study was conducted to evaluate the usefulness of OGEL as a sublingual vaccine carrier against Chlamydia. Thus, mice were administered CTH522 in CAF01 subcutaneously or followed by a sublingual boost. In addition, sublingual vaccination alone was also evaluated. We established parenterally immunized animals with CAF01/CTH522 as the control group due to naïve animals did not show CTH522-specific antibodies and cytokines (Figures S1 and 2S, Supporting Information). **Figure 3** shows total IgG and IgA CTH522 specific antibodies determined in serum (A, B) and vaginal lavage (C, D) at the end of the study. Serum results showed that mice receiving mucosal boosting with antigen alone or loaded in OGEL after subcutaneous antigen stimulation, produced lower CTH522-specific IgG antibody levels than their counterparts only immunized parenterally. Nevertheless, parenterally primed and sublingually boosted animals exhibited higher IgA responses both in serum and vaginal washes,

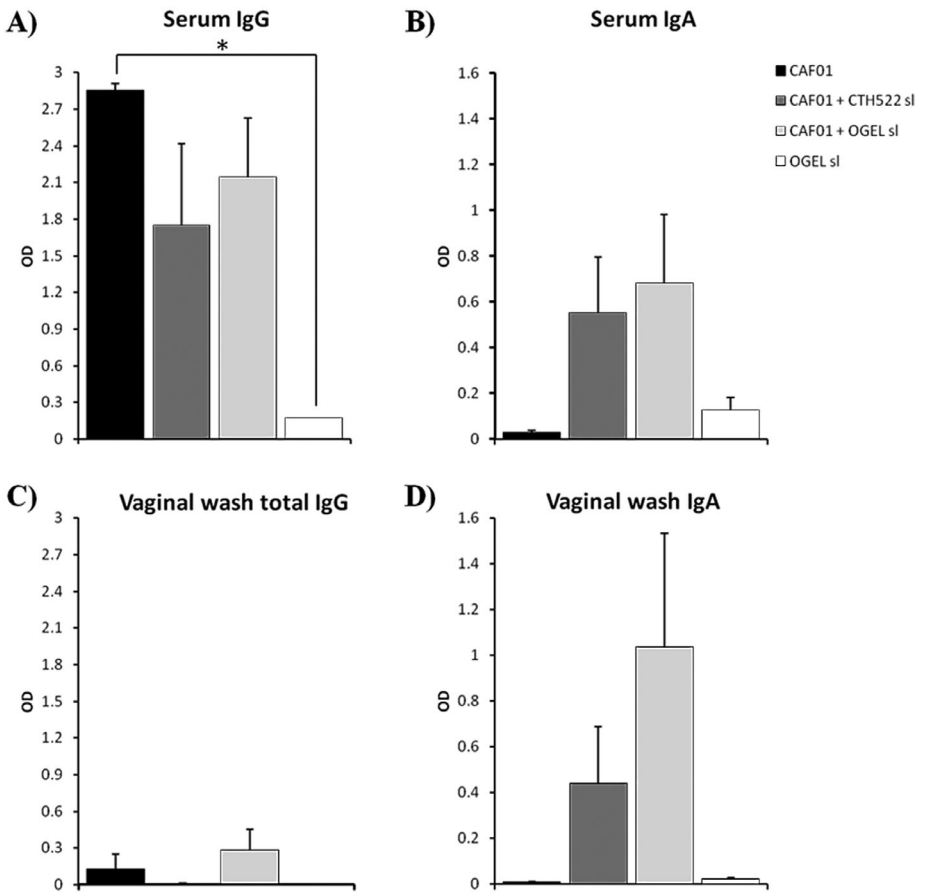

Figure 3. Total IgG and IgA determined by ELISA in serum A, B) and vaginal lavage C, D). The y-axis shows the optical density (OD). Results are expressed as mean ± SEM. * denotes statistically significant differences between depicted groups ($p < 0.05$).

particularly when loaded on the hydrogel (OGEL/CTH522). These results are consistent with previous studies that describe the boosting effect of the sublingual route.[14,40,41] Animals vaccinated parenterally with CAF01/CTH522 had significantly higher serum IgG responses than mice immunized exclusively by the sublingual route ($p < 0.05$) (**Figure** 3A–D). Although the group boosted with OGEL/CTH522 produced the highest IgA and IgG mean titers in vaginal secretions, which is one of the vaccination targets against sexual diseases, this enhancement was not statistically significant compared to animals only vaccinated with CAF01/CTH522.

3.3. Prime Immunization Using CAF01 followed by OGEL Sublingual Boosting Elicits High-Magnitude IFN-γ Responses in Cervical Lymph Nodes

Optimal vaccination against *Chlamydia trachomatis* requires stimulating Th1 cell-mediated immunity in addition to eliciting neutralizing antibodies. Th17 cells have a crucial role as accelerators of mucosal immunity. Besides, these cells can also acquire functional features of follicular helper T cells, which can lead to IgA-isotype switching.[3] To measure if sublingual boosting delivered in OGEL can improve local and systemic Th1 and Th17 responses, we re-stimulated splenocytes and cervical LN cells with CTH522 antigen and measured secretion of IFN-γ and IL-17A. As shown in **Figure 4**A–D, the secretion of IFN-γ by splenic T cells was higher than IL-17A, while the opposite was seen in cervical lymph nodes. Generally, mucosal boosting was beneficial to improve IFN-γ and IL-17A responses in both spleen and cervical lymph nodes after parenteral priming. Splenocytes from the group having received parenteral prime/loaded hydrogel sublingual boost tend to secrete a higher amount of IFN-γ, although no statistically significant differences were obtained when compared to the group that received only parenteral vaccine. However, in cervical lymph nodes, IFN-γ production was significantly higher in the group that had received parenteral prime/loaded hydrogel sublingual boost compared to parenteral vaccine alone ($p < 0.05$) (Figure 4C). Using a mucoadhesive hydrogel as a carrier of CTH522 vaccine for sublingual boosting enhanced the secretion of IFN-γ and IL-17A in both spleen and cervical lymph nodes. Specifically, boosting with OGEL/CTH522 showed a two-fold and 1.4-fold increase of IFN-γ and IL-17A secretion respectively in the spleen when compared to mucosal boosting with CTH522 alone. The same effect was observed in cervical lymph node samples, where animals boosted with OGEL/CTH522 presented a 3.2 and 4.3-fold enhancement in IFN-γ and IL-17A, respectively, compared to those receiving CTH522 alone, although these differences were not statistically significant. Overall, the systemic prime-sublingual boost strategy was more effective in

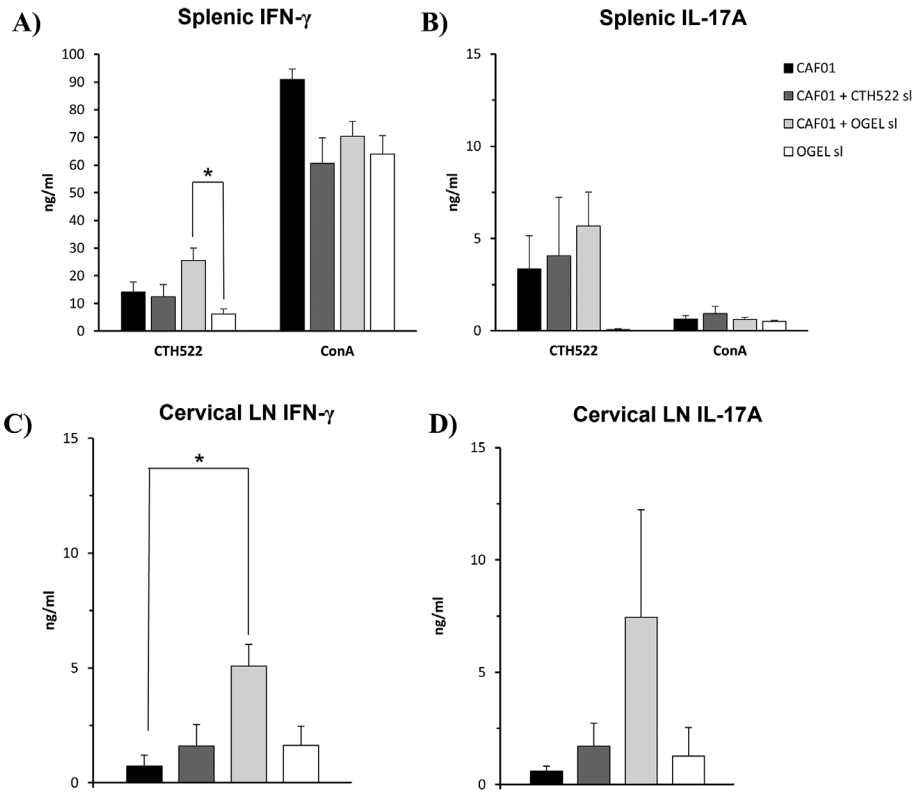

Figure 4. IFN-γ and IL17A cytokine production by splenocytes A, B) and cervical lymph nodes cells C, D) after being restimulated with CTH522 or ConA (positive control). Results are expressed as mean ± SEM. * denotes statistically significant differences between depicted groups ($p < 0.05$).

inducing Th1/Th17 immune responses than parenteral priming alone, and boosting with antigen formulated in OGEL may further increase responses.

4. Discussion

The use of mucosal tissues as a vaccination route is grounded in the fact that most infections are initiated at the mucosal surfaces.[42] Novel vaccination strategies are mainly focused on subunit vaccines, including recombinant peptides and proteins, DNA, mRNA, or toxoids, owing to their higher safety profile and cheaper mass production. Nevertheless, these types of vaccines present the great disadvantage of being poorly immunogenic.[5,43] This drawback can be even more pronounced when administered mucosally due to antigen dilution and/or entrapment in mucosal secretions and proteolytic degradation, hindering antigen uptake and delivery to the mucosa-associated lymphoid tissues.[5] In this sense, adjuvants and vaccine delivery platforms become crucial for achieving effective and long-lasting immunity.

Parenteral vaccines may be effective when the infection occurs in the urogenital tract because it is more permeable to the transudation of serum antibodies than other mucosae. The antibody titers however must be high,[23] and so parenteral vaccines frequently fail in inducing strong antibody responses at mucosal tissues.

The promotion of high IgA titers able to neutralize pathogens at the target mucosal surface(s) is extraordinarily challenging, even when a mucosal tissue is used as an immunization site.[44] Several studies have hallmarked the synergistic effect of combined systemic and mucosal vaccination to generate strong local mucosal immunity and protective immunity either in mice and minipigs against Chlamydia[3,15,16,23] or in macaques against simian immunodeficiency virus.[45] Besides, the benefits of parenteral priming followed by mucosal boosting were also described for other mucosal pathogens such as *Mycobacterium tuberculosis*.[46,47] In this work mice were primed subcutaneously with the chlamydia subunit vaccine CTH522 incorporated in CAF01 followed by two mucosal boosting using the sublingual route to evaluate the potential synergic effect of both routes. The comparison between the two sublingual boosting strategies (antigen alone or embedded in a hydrogel) should allow us to assess the utility of a new hydrogel platform to efficiently promote mucosal immune responses.

Sublingual vaccination can induce IgA responses with similar mucosal tropisms to nasal vaccination, but is generally considered less immunogenic.[44] In an attempt to maximize CTH522 uptake by the sublingual mucosa and improve mucosal and systemic immune responses, the antigen was loaded on the thermosensitive and mucoadhesive formulation OGEL.

The liquid texture of OGEL facilitates its application and extension on the sublingual mucosa, while its thermosensitive properties allow a fast sol-gel transition at body temperature, avoiding formulation leakage by swallowing. Furthermore, a highly mucoadhesive hydrogel should increase the contact time with the sublingual epithelium and facilitate antigen uptake. These properties make OGEL a good candidate as a vaccine carrier

for this route of administration.[22,48,49] OGEL is a ternary system whose components have interesting properties. PF127 has been reported to enhance drug permeation through different mucosae and could therefore potentially increase CTH522 permeation through sublingual tissue.[22,50] HS1200 exhibited potential adjuvant properties by promoting the proliferation of human macrophages.[20] Lastly, Gantrez is a high bioadhesive anionic amphiphilic copolymer, previously used in oral vaccines as an excipient of nanoparticles or microneedle patches.[51–53]

Previous studies have used CAF01 as a vaccine adjuvant against Chlamydia,[3,23,54,55] reporting its capability for inducing immune responses with a Th1/Th17 profile together with high antibody titers. IFN-γ produced by Th1 cells plays a key role in the resolution of chlamydia infection, inhibiting Ct growth through different pathways.[56–58] Th17 cells have not been associated with a direct role against Chlamydia, but stimulate neutrophil recruitment and are involved in the development of Th1 immunity[59] and production of mucosal IgA.[3]

Our results point to a synergistic effect of parenteral and sublingual vaccination, as mice immunized by the two routes produced higher IgA titers and developed stronger cellular responses in splenocytes and cervical lymph nodes than when using a single route. Similar results were observed in mice simultaneously vaccinated intramuscularly and intranasally with *Chlamydia psittaci* antigens, adjuvanted with chitosan nanoparticles.[60] Our results are also in agreement with studies by Ralli-Jain et al.[16] and Carmichael et al.,[15] using mice immunized with the recombinant major outer membrane protein (rMOMP) of Ct and *Chlamydia muridarum*, respectively.

The results herein described that sublingual boosting after systemic priming could be a feasible strategy to induce systemic as well as mucosal immunity in the genital tract. In addition, our results suggest that the use of mucoadhesive vaccine delivery systems could be a strategy to maximize the boosting effect of sublingual immunization and therefore, improve mucosal immune responses in the genital tract. However, the success of this approach will depend on the development of highly mucoadhesive vaccine carriers capable of promoting a fast antigen permeation through sublingual tissue, thus ensuring an adequate stimulation of the immune system. Sublingual administration of CTH522 loaded OGEL after subcutaneous CAF01/CTH522 priming elicited IgA responses in serum and vaginal fluids, as well as IFN-γ and IL-17A secretion by spleen and cervical lymph node T cells.

Mouse oral anatomy differs from that of humans, particularly regarding the sublingual mucosa, which is highly keratinized in mice. High levels of keratinization strongly hinder the permeability of active substances across tissues.[22,61] This limitation must be considered when these rodents are used as a model for testing the usefulness of drugs or vaccine systems by sublingual administration. Animal models with greater similarity to humans, such as primates, would be better suited to predict immune responses, but costs and ethical issues limit their use.[62]

5. Conclusions

ANN together with genetic algorithms has demonstrated its potential to optimize a ternary hydrogel formulation through a reduced experimental design, giving rise to OGEL, a highly mucoadhesive and thermosensitive hydrogel suitable as a vaccine carrier for sublingual administration. OGEL has shown utility as a carrier of the chlamydia vaccine CTH522 in a mouse model. The co-administration of CAF01 and OGEL loaded with CTH522 subunit vaccine significantly enhanced IFN-γ production by cervical lymph nodes when compared with mice exclusively immunized parenterally.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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cationic adjuvant liposomes, mucoadhesive and thermosensitive platforms, mucosal vaccination, polymeric networks, prime-boost strategy, systemic and sublingual immunization

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