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CAF01 liposomes as a mucosal vaccine adjuvant: *In vitro* and *in vivo* investigations

Dennis Christensen[a,*], Camilla Foged[b], Ida Rosenkrands[a], Carina Vingsbo Lundberg[c], Peter Andersen[a], Else Marie Agger[a], Hanne Mørck Nielsen[b]

[a] *Statens Serum Institut, Department of Infectious Disease Immunology, Vaccine Delivery & Formulation, Artillerivej 5, DK-2300 Copenhagen S, Denmark*
[b] *University of Copenhagen, The Faculty of Pharmaceutical Sciences, Department of Pharmaceutics and Analytical Chemistry, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark*
[c] *Statens Serum Institut, National Center for Antimicrobials and Infection Control, Artillerivej 5, DK-2300 Copenhagen S, Denmark*

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ABSTRACT

Mucosal administration of vaccines has many advantages compared to parenteral vaccination. Needle-free mucosal vaccination would be easily applicable, target the vaccine to the entry point of many pathogens, and reduce the risk of infection with other pathogens during vaccination as compared to invasive methods.

CAF01 is a novel liposome-based vaccine adjuvant with remarkable immunostimulatory activity. The potential of CAF01 liposomes as adjuvant for mucosal vaccines was investigated using the Calu-3 epithelial cell culture *in vitro* model. Thus, the mucosal permeability of the antigen as well as the epithelial integrity and the metabolic activity of the well-differentiated cells were investigated after exposure to CAF01. Finally, the adjuvant was tested for nasal administration in mice, combined with an influenza vaccine.

The results suggest that CAF01 enhanced transport of antigen through the mucus layer on Calu-3 cells, increasing the concentration of antigen in the cell layer, as well as the transport across the epithelial cells. Furthermore CAF01 was well tolerated by the Calu-3 cells and the *in vivo* studies demonstrated increased cell-mediated immunity (CMI) as well as humoral immune responses in mice after nasal application of the influenza vaccine when combined with CAF01. CAF01 is thus a promising adjuvant for mucosal delivery.

1. Introduction

Vaccines are widely regarded as cost effective tools for improving health, and have for the last century had a major impact on public health. It is estimated that vaccines annually prevent almost 6 million deaths worldwide (Ehreth, 2003a,b).

Parenteral administration of vaccines, however, possesses safety risks for patients and health care providers. In fact, the number of hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infections among health care professionals attributable to sharps injuries are estimated to 83,000 incidences per year (Prüss-Üstün et al., 2005).

Non-invasive routes of administration are therefore interesting alternatives, and mucosal application has great potential. Mucosal immunity provides the first line of defence against pathogens such as influenza virus, mycobacteria and chlamydia that infect the host via the mucosa. Such pathogens are mainly cleared non-immunologically by the first defence layer of the mucosa consisting of mucus, ciliated epithelial cells and enzymes. Immunological defence mechanisms, such as the mucosa-associated lymphoid tissue (MALT), exist to eliminate pathogens if they bypass the first defence layer.

The majority of novel vaccination strategies are based on recombinant protein antigens, which by themselves are very poor immunogens upon mucosal administration due to fast clearance at mucosal sites. These vaccine antigens therefore require a delivery vehicle, as well as an immunostimulator, in order to be recognised and taken up by the immune system, with concomitant immune activation. CAF01 is a novel vaccine adjuvant system, based on liposomes formed by the cationic surfactant dimethyldioctadecylammonium bromide (DDA) and the immunostimulator trehalose 6,6′-dibehenate (TDB). This adjuvant has a remarkable immunostimulatory activity, characterized by induction of both cell-mediated (CMI) and humoral immune responses when administered parenterally (Davidsen et al., 2005). Furthermore, immunization by injection has shown significant levels of protection with selected candidate vaccine antigens co-administered with CAF01 in three animal disease models with markedly differ-

* Corresponding author. Tel.: +45 32 68 38 04.
E-mail address: den@ssi.dk (D. Christensen).

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ent immunological requirement; *Mycobacterium tuberculosis* (CMI), *Chlamydia trachomatis* (CMI/humoral) and blood-stage malaria (humoral) (Agger et al., 2008).

CAF01 liposomes are highly polydisperse multivesicular vesicles with an average particle size of approximately 450 nm. The liposomes are strongly cationic with a surface charge around +60 mV and have a phase transition temperature above 37 °C, which means that the lipid membrane of CAF01 is in the solid ordered state at physiological temperature (Davidsen et al., 2005; Christensen et al., 2007).

The purpose of this study was to investigate the potential of CAF01 liposomes as an adjuvant system for mucosal vaccines and to investigate the effect of CAF01 on mucosal epithelial cell viability. The human bronchial epithelial Calu-3 cell culture model produces mucus and forms a ciliated cell layer, which makes it a suitable model for the upper airways. It was therefore chosen as a model system to elucidate the epithelial deposition, absorption, and retention of the antigen upon administration in a liposomal adjuvant system. The effect of CAF01 on cell viability was investigated by analyzing the epithelial integrity of the cell layer(s) and the metabolic activity of the Calu-3 cells. Finally, the adjuvant was tested nasally in a mouse model using a commercially available trivalent influenza split vaccine, and antigen-specific interferon (IFN)-γ production by spleen cells and serum IgG levels were determined.

2. Materials and methods

2.1. Materials

Dimethyldioctadecylammonium (DDA) bromide and α,α′-trehalose 6,6′-dibehenate (TDB) were obtained from Avanti Polar Lipids (Alabaster, AL). The purity of the compounds was >99% by high performance liquid chromatography (HPLC). Methanol (extra pure) and chloroform (extra pure) were purchased from Merck (Darmstadt, Germany). Ovalbumin (OVA), ^{14}C-methylated ovalbumin (^{14}C-OVA, specific activity 40 μCi/mg), ^{3}H-labelled mannitol (specific activity 20 Ci/mmol) and tris base (99%) were obtained from Sigma–Aldrich (St. Louis, MO). GIBCO® Hank's-Buffered Salt Solution (HBSS) was obtained from Invitrogen (Carlsbad, CA). Purified water of Milli-Q quality was used to prepare all buffers. Vaxigrip 2005/06 influenza split vaccine was acquired from Sanofi Pasteur MSD (Lyon, France).

2.2. Preparation of adjuvant formulations

The adjuvant was prepared by the thin film method as described previously (Davidsen et al., 2005). Briefly, weighed amounts of DDA and TDB (5:1, w/w) were dissolved in chloroform/methanol (9:1, v/v) and the organic solvent was removed using a gentle stream of N_2, forming a thin lipid film on the bottom of the test vial. The lipid film was dried over night under vacuum to remove trace amounts of the organic solvent. The lipid film was hydrated in the 10 mM tris buffer (pH 7.4) by heating for 20 min at 10 °C above the main phase transition temperature of DDA (Tm ≈ 47 °C) (Feitosa et al., 2000) to a final concentration of 2.5 mg/ml DDA and 0.5 mg/ml TDB for the Calu-3 cell assays resulting in a final application dose of 0.25 mg DDA and 0.05 mg TDB. The final concentration for the intranasal (*i.n.*) vaccines was 3.25 mg/ml DDA and 0.625 mg/ml TDB resulting in a final application dose of 0.125 mg DDA and 0.025 mg TDB.

2.3. Calu-3 cells

The Calu-3 cell line (HTB-55) was obtained from the American Type Culture Collection (ATCC; Rockville, MD), and it was cultured in T-75 culture flasks (Corning Costar, Biotech line, Denmark) in an atmosphere of 5% CO_2–95% O_2 at 37 °C and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich, Denmark) containing 10% (v/v) fetal bovine serum (Gibco, Biotech line, Denmark), 90 U/ml penicillin and 90 μg/ml streptomycin (BioWhittaker, Cambrex, Denmark). Cells were sub-cultured at 90% confluence. Cells were seeded at a density of 9×10^4 cells/insert onto 12-well polycarbonate Transwell filters (0.9 cm^2 growth area, 0.4 μm pore size; Corning Costar, Biotech line, Denmark) coated with collagen (0.30 μg/ml, 200 μl/insert; VWR, Denmark) and grown at air-interface conditions. The cells were allowed to attach for 24 h before removal of the medium in the apical compartment. The monolayer was then allowed to differentiate under air-interface feeding conditions by replacing the culture medium in the basolateral compartment every other day for 2 weeks. Passage numbers 39, 41 and 48 were used in the studies.

2.4. Deposition, absorption and retention

The culture medium was removed, and the cells were washed with 0.5 and 1 ml HBSS on the apical and basolateral side, respectively, and pre-equilibrated to room temperature in order to measure the pre-incubation transepithelial electrical resistance (TEER). After removal of the HBSS, 110 μl test solution containing either 250 nCi ^{3}H-mannitol or 50 nCi ^{14}C-OVA (adjusted to 2 μg/dose with non-labelled OVA) was applied to the apical side of the cell model in the absence or presence of 300 μg/dose CAF01 corresponding to the normal murine vaccination dose (Davidsen et al., 2005; Agger et al., 2008). The filter inserts were immediately transferred to HBSS-containing wells (1 ml) placed on a horizontal shaker pre-set at 37 °C. Receptor samples of 100 μl were withdrawn from the basolateral side at 0, 20, 60, 120, 180 and 240 min after sample application and transferred to scintillation vials. The sample volume taken from the receptor side was replaced with 100 μl HBSS. After 240 min, the test solutions were removed from the filters and 0.5 ml HBSS was added to the apical side. After adjustment to room temperature, the post-incubation TEER was measured. The filters were separated from the inserts and transferred to scintillation vials to investigate the deposition of the antigen in the cell layer. The mucus was removed from a number of the filters before transfer of the filters to the scintillation vials. Quantification of ^{3}H-mannitol and ^{14}C-OVA samples was done by liquid scintillation (TRI-CARB 2100 TR, Packard Instruments, Meriden, CT). The apparent permeability coefficient of mannitol and OVA was determined in the donor to receptor direction according to:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0 \times 60} \quad (1)$$

where dQ/dt is the flux at steady state, A is the diffusion area (0.9 cm^2) and C_0 is the initial donor concentration. dQ/dt was determined by plotting the cumulative amount of mannitol/OVA in the receptor compartment (Q) against time (t) where

$$Q = V_s \times \left(\sum_{n=1}^{n} C_{n-1}\right) + C_n \times V_t \quad (2)$$

and V_s is the withdrawn volume (100 μl), V_t is the total receptor volume (1 ml), C_n is the concentration in the receptor compartment at time n and $\sum_{n=1}^{n} C_{n-1}$ is the sum of concentrations in the receptor samples taken before time n. The enhancement ratio (ER) was determined as the relation between P_{app} values for ^{3}H-mannitol/^{14}C-OVA in the presence of CAF01 relative to in the absence of CAF01.

2.5. Integrity (TEER)

The TEER of the epithelial monolayer was measured in HBSS at room temperature both pre and post incubation with test solution

HBSS using an Endohm tissue resistance measurement chamber coupled to an EVOM™ epithelial volt-ohmmeter (World Precision Instruments, FL, USA). The relative TEER value is given as the post-incubation TEER relative to the pre-incubation TEER.

2.6. Viability (MTS/PMS assay)

After finalizing the cell culture experiment, a volume of 320 μl MTS/PMS reagent was added to the apical side of the epithelium with 1.0 ml HBSS present at the basolateral side. The cells were incubated on a horizontal shaker for 1 h at 37 °C. Two times 100 μl samples were then withdrawn from the apical side of each filter and transferred to clear 96-well plates and the absorbance was measured at 492 nm on a FLUOstar OPTIMA plate reader (BMG Labtech GmBH, Offenburg, Germany) (Eirheim et al., 2004).

2.7. Immunization of mice

Female BALB/c mice, 8–10 weeks old (Harlan Scandinavia, Allerød, Denmark) were housed at the animal facility at Statens Serum Institut. The mice had ad libitum access to domestic quality drinking water and food (2016 16% Protein Rodents Diet, Harlan, USA). The mice were housed in Type 3 macrolone cages with 4–8 mice/cage. The vaccines were prepared by mixing the CAF01 with the Vaxigrip (Sanofi Pasteur MSD), season 2005/06 influenza vaccine. The final amount of influenza vaccine was 1 μg/dose, and the final amount of CAF01 was chosen as 150 μg/dose, for all formulations, which is half of the normal murine injected dose, due to limitation in the small *i.n.* vaccination volume (40 μl/dose) as compared to s.c. and i.m. vaccination (200 and 100 μl/dose, respectively). The mice ($n = 6$) were vaccinated *i.n.* with 20 μl in each nostril at day 0, 1, 19 and 21. All animal experiments were approved by the National Committee of Animal Ethics–Animal Experiment Inspectorate under the Danish Ministry of Justice.

2.8. Detection of vaccine-specific IFN-γ cytokines by ELISA

Spleens were obtained from mice 7 weeks after the last immunization and prepared as previously described (Rosenkrands et al., 2005). Cell cultures were performed in triplicate in round-bottomed microtiter wells, containing 2×10^5 cells in a volume of 200 μl RPMI supplemented with 5×10^{-5} M2-mercaptoethanol, 1 mM glutamine, 1% penicillin–streptomycin, 1% HEPES, and 10% foetal calf serum (all from Gibco, Invitrogen, Carlsbad, CA). Cells were re-stimulated with the influenza split vaccine at a concentration of 1 μg/ml. Wells containing medium only or 5 μg/ml of Concanavalin A (Sigma–Aldrich, St. Louis, MO) were included in all experiments as negative and positive controls, respectively. Culture supernatants were harvested from parallel cultures after 72 h of incubation in the presence of antigen, and the amount of IFN-γ was determined by enzyme-linked immunosorbent assay (ELISA) (Billeskov et al., 2007).

2.9. Detection of vaccine-specific antibodies by ELISA

Microtiter plates (Nunc Maxisorp™, Roskilde, Denmark) were coated with influenza split vaccine (1 μg/ml) in carbonate-buffer pH 9.6 overnight at 4 °C. Free binding sites were blocked with 2% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS). Individual mouse sera from six mice per group were analysed individually in five-fold dilutions at least 7 times in PBS containing BSA starting with a 100-fold dilution. Horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit anti-mouse immunoglobulin G1; Zymed, San Francisco, CA) diluted 1/5000 in PBS with 1% (w/v) BSA were added. After 1 h of incubation, antigen-specific antibodies were detected by 3,3′,5,5′-tetramethylbenzidine (TMB) substrate as described by the manufacturer (Kem-En-Tec, Copenhagen, Denmark). The antibody log EC_{50} values were calculated in GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA) by fitting the pre-grouped titration results into a sigmoidal dose-response model.

2.10. Statistics

For comparative analysis of deposition, integrity and immune responses, data were tested by one-way analyzes of variance (ANOVA, if more than two groups) or a *T*-test (if two groups) with a 95% confidence interval assuming that the data had a Gaussian distribution. For comparative analysis of viability results, data were tested by two-way ANOVA covering treatment groups and assay dilution. When significant differences were indicated, differences between means were determined by Bonferonni's multiple comparison tests. All statistical analyses were performed in GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. CAF01 increases the permeability through mucus and epithelium

Mannitol is metabolically inert, small and highly hydrophilic and has previously been used to trace changes in the epithelial integrity of mucosal cell models. It diffuses poorly into the cell membranes, but more easily through the aqueous paracellular pathway. Mannitol is therefore an ideal model compound to detect changes in paracellular permeability of epithelia. In this study, mannitol was used as an internal control and as illustrated in Fig. 1, permeated the Calu-3 epithelium with a linear concentration/time relationship. P_{app} was calculated from the measured steady state flux rate according to Formula (1). P_{app} for mannitol + CAF01 was significantly higher than for mannitol alone and the enhancement ratio of CAF01 was calculated to $ER = 3.36$ ($n = 6$).

An increase in OVA absorption across the Calu-3 monolayer from the apical to the basolateral side was observed when co-administering CAF01 (Fig. 2A). The permeability of OVA did not follow Fick's first law of diffusion. This is illustrated in Fig. 2A, where the cumulative permeability as a function of time is not linear, whereas the diffusion of mannitol as expected is constant over time (Fig. 1A). The reason for this discrepancy could be (1) that the permeability of OVA does not occur via passive diffusion, (2) that the apical concentration accessible for permeation is not constant due to complexation in the mucus layer, or (3) that OVA is lost from the bulk volume at the basolateral side, maybe by adsorption to the walls of the well. It is though beyond the scope of this study to understand the permeability kinetics of OVA through mucus and epithelium. The P_{app} was therefore calculated according to formula (1), based on the steady state flux after 60 min. P_{app} for OVA in the presence of CAF01 was significantly higher when compared to OVA alone (Fig. 2B). The enhancement ratio of OVA transport upon applying CAF01 was 1.31 ($n = 9$). The total amount of OVA permeated across the epithelium, however, made up as little as 4.3% and 5.3% of the applied amount when administered in the absence or presence of CAF01, respectively. The amount of OVA found in the epithelial cell layer upon removal of the mucus was therefore also measured and results ($n = 3$) showed that approximately 22.1% of the applied OVA was found in the cell layer upon administration together with CAF01, whereas only 4.1% was found in the cell layer when administered alone (Fig. 2C), suggesting that there was an increased diffusion of the antigen through the mucus when co-administered with CAF01.

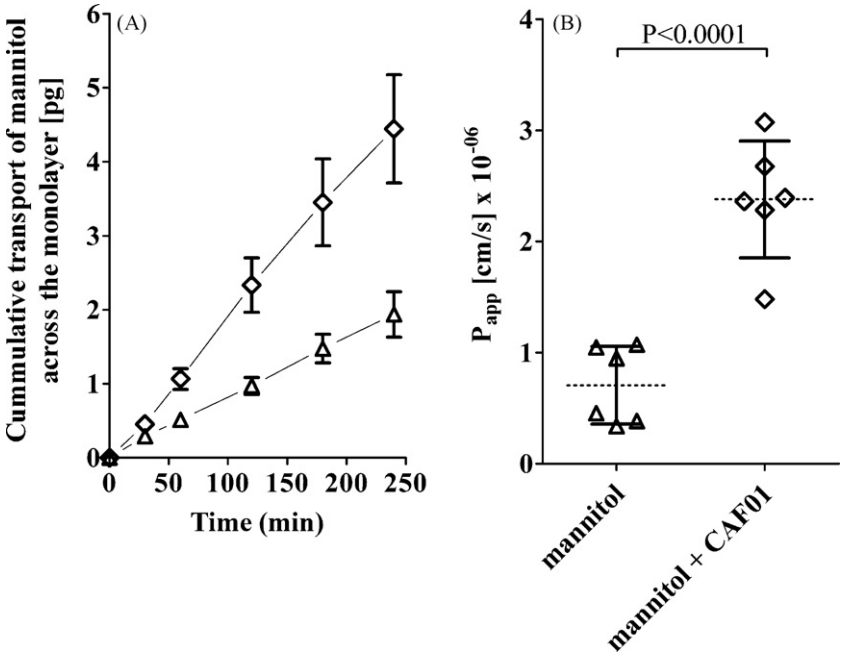

Fig. 1. (A) Cumulative transport of mannitol (△) and mannitol + CAF01 (◊) with time ($n = 3$, mean ± SD). (B) Calculated P_{app} for mannitol (△) and mannitol + CAF01 (◊) the dotted line shows the mean ± SD. The mean P_{app} of mannitol was 0.71×10^{-6} cm/s ($n = 6$, SD = 0.35×10^{-6}) and the mean of mannitol + CAF01 was 2.38×10^{-6} ($n = 6$, SD = 0.53×10^{-6}).

3.2. CAF01 does not decrease the integrity and viability of the Calu-3 cells

TEER was monitored in order to assess the effect of the model vaccines on the integrity of the tight junctions between the Calu-3 cells. The TEER value of the epithelium on all filter inserts was measured prior to application of the ^{14}C-OVA in the absence or presence of CAF01 and after termination of the study. The TEER value of the Calu-3 cells before vaccine application was $322 \pm 54\ \Omega\ cm^2$ corresponding to standard values in our laboratory, and confirming the integrity of the epithelium since the minimal TEER value needed for a tight monolayer has been reported to be $129\ \Omega\ cm^2$ for Calu-3 cells cultured on 0.4 μm pore filters (Geys et al., 2007). The TEER values of the epithelial layers after 4 h of exposure to mannitol or OVA in the absence or presence of CAF01 all increased with approximately 50% (Fig. 3A). There was, however, no significant difference between the obtained relative TEER values suggesting that neither mannitol nor OVA alone or in combination with CAF01 affected the integrity of the tight junctions, and thereby the barrier properties of the Calu-3 epithelium. A general increase in TEER was observed in all groups, including the controls exposed to only mannitol. This is probably explained by a difference in the temperature prior to TEER measurements rather than a change in epithelial integrity during the experiment. In that case, non-linearity in Fig. 1A would have been expected. In order to test whether the mucus layer facilitated protection of the epithelium, it was removed prior to the experiments. This did not affect the integrity of the Calu-3 epithelium, suggesting that CAF01 does not have any influence the integrity of the mucus depleted epithelium (data not shown).

Using the MTS/PMS assay, the dehydrogenase activity in the Calu-3 cells was measured to estimate the differentiated cell sensitivity to OVA with and without simultaneous addition of CAF01 as an indication of cell viability. Fig. 3B shows the dilution curves for absorbance of formazan, a reduction product of MTS made by

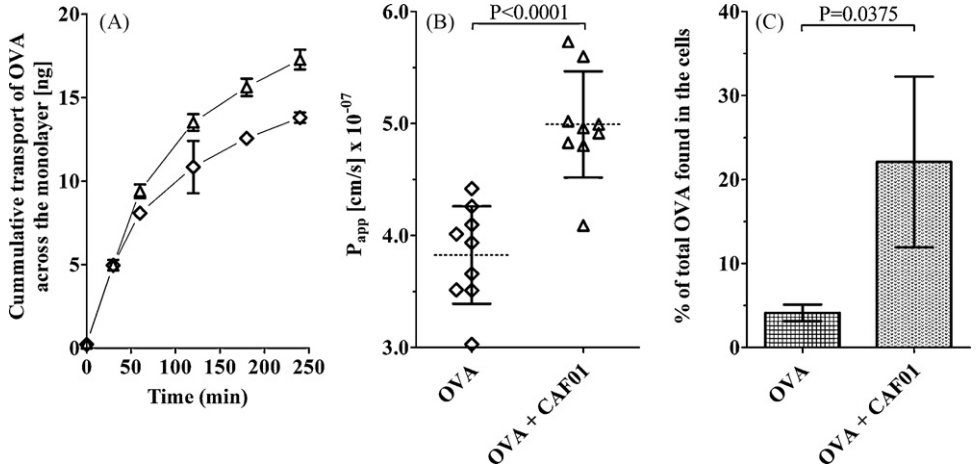

Fig. 2. (A) Cumulative transport of OVA (◊) and OVA + CAF01 (△) over time ($n = 9$, mean ± SD). (B) Calculated P_{app} for OVA (◊) and OVA + CAF01 (△). The dotted line shows the mean ± SD. The mean of OVA was 2.97×10^{-6} cm/s ($n = 9$, SD = 0.34×10^{-6}) and the mean of OVA + CAF01 was 3.86×10^{-6} cm/s ($n = 9$, SD = 0.37×10^{-6}). (C) The % of total OVA found in the epithelial cell layer after removal of mucus. The mean amount of OVA found in the cell layer constituted 4.1% ($n = 3$, SD = 0.40) of the total amount, whereas the mean amount of OVA in the presence of CAF01 found in the cell layer was 22.10% ($n = 3$, SD = 10.16) of the total amount.

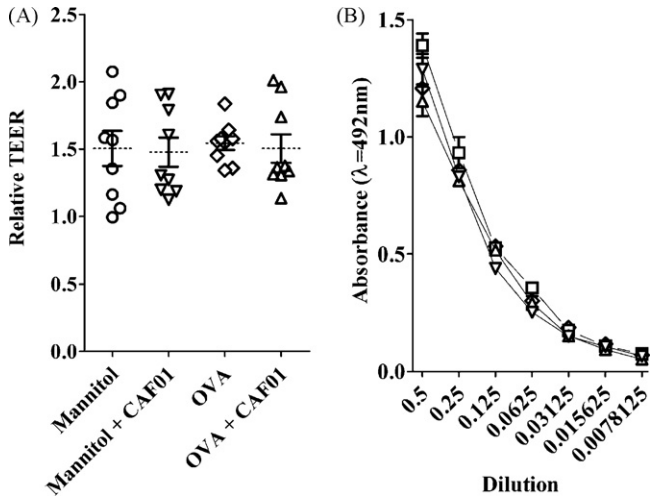

Fig. 3. (A) The relative TEER was determined by subtracting the pre-exposure TEER from the post-exposure TEER ($n = 9$ over 3 separate passages). ANOVA showed that there was no significant difference between the three groups ($P = 0.9501$). (B) The light absorption of formazan after dilution of samples taken from apical side of unexposed cells (◊) or cells exposed to OVA (□), CAF01 (△) or OVA + CAF01 (▽). A two-way ANOVA showed that there was no significant difference between the light absorbance of the untreated group and the OVA and/or CAF01 treated groups and that the absorbance accounts for 0.42% of the total variance ($P = 0.0669$).

mitochondrial reductase enzymes. There was no significant difference in the absorbance, and thus the amount of MTS reduced by the cells, when comparing HBSS-exposed control cells with cells exposed to OVA in the absence or presence of CAF01 or CAF01 alone. This implies that there is no difference in the mitochondrial activity of the epithelial cells irrespective of whether they are exposed to OVA and/or CAF01.

3.3. CAF01 significantly increases the immune response towards influenza antigens after i.n. vaccination

The *in vivo* adjuvant effect of CAF01 was investigated by immunizing mice intra-nasally with a commercially available influenza split vaccine. Adding CAF01 to the vaccine formulation significantly increased the vaccine-specific immune response. The antigen-specific IFN-γ response in spleens from mice vaccinated with the CAF01 formulation was 4 times higher than in mice vaccinated with the non-adjuvated formulation (Fig. 4A). The vaccine specific serum IgG level was also significantly enhanced by adding CAF01 to the vaccine formulation (Fig. 4B and C).

4. Discussion

The vast majority of pathogens enters and/or colonizes the organism via the mucosa. Mucosal immunization would therefore be of major interest for fast elimination of these pathogens. One concern, however, is that the mucosa mainly consists of a vulnerable epithelium with secretion of viscous mucus, which acts as a first barrier against pathogens. A vaccine should therefore be able to penetrate the mucus effectively and, without damaging the epithelial cells, penetrate into or permeate across the epithelium to interact with circulating antigen presenting cells or interact directly with the MALT.

In this study, we investigated the effect of the liposome-based vaccine adjuvant CAF01 on the antigen absorption through and retention in human airway epithelial cells and the mucus produced by this cell layer, using the Calu-3 cell model. The Calu-3 cell line is a useful *in vitro* model for comparative investigations and for mechanistic studies of vaccine absorption by the airway epithelium (Foster et al., 2000; Florea et al., 2003). Monolayers of the polarized Calu-3 cells differentiate into a phenotype that displays physiological features of the upper airway epithelium, such as mucus production and cilia, which are relevant for drug and vaccine delivery. The results in this study showed that CAF01 increased the transepithelial absorption of a model protein antigen, ^{14}C-OVA, however, only from 4.3% to 5.3% over 4 h of the total amount applied. The Calu-3 epithelium was hence isolated from the mucus in order to localize the remaining ^{14}C-OVA, and 22.1% of the total amount of OVA was found in the cell layer when administered together with CAF01, whereas only 4.1% was found in the cell layer upon administration without CAF01. This indicates that CAF01 enhances the antigen transport through the mucus layer,

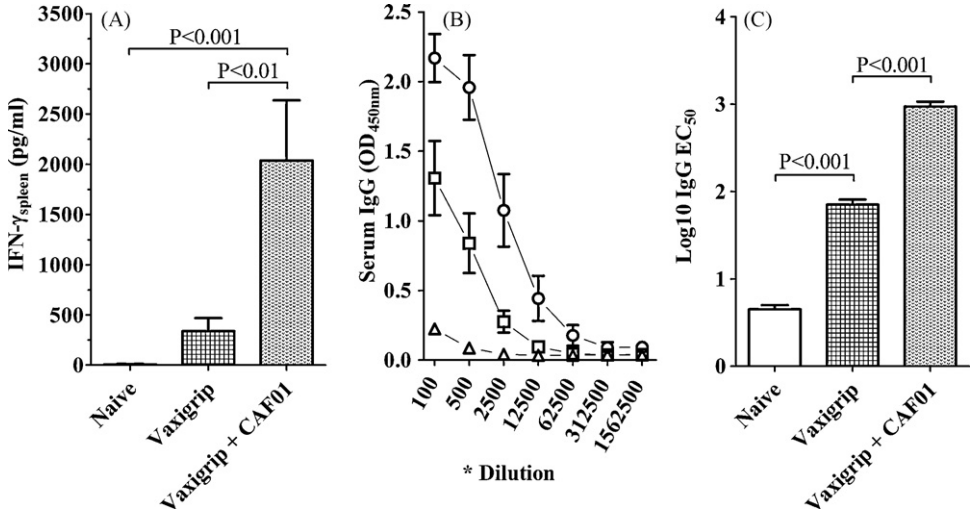

Fig. 4. BALB/c mice ($n = 6$) were immunized four times with 1 μg Vaxigrip 2005/06 alone or with CAF01. (A) Individual cultures of splenocytes were harvested 7 weeks after the last immunization and restimulated *in vitro* with Vaxigrip 2005/06 vaccine. The release of IFN-γ was determined by ELISA. ANOVA showed that the IFN-γ responses after vaccination with the adjuvated vaccine were significantly higher than the control group ($P < 0.001$) and vaccination with Vaxigrip alone ($P < 0.01$). The IFN-γ responses induced by Vaxigrip alone were not significantly higher than those found in the unvaccinated mice. (B) Sera were analysed for the presence of antigen-specific IgG antibodies by ELISA after no vaccination (△) and after vaccination with Vaxigrip alone (□) or Vaxigrip in CAF01 (○). (C) ANOVA analysis of EC50 values showed that the IgG responses after vaccination with the adjuvated vaccine were significantly higher than the control group ($P < 0.001$) and that vaccination with Vaxigrip alone induced an IgG response, which was significantly higher than that found in the unvaccinated mice ($P < 0.001$).

rather than through the cell layer. For vaccination purposes, this would be beneficial since the target would be the MALT, which would hence experience an increased exposure to the antigen besides potentially being stimulated as a result of the adjuvant effect of CAF01. The enhanced antigen transport is most probably due to association of the negatively charged OVA with CAF01 having a positive surface charge. This association was observed not to result in any precipitation or aggregation in the test samples applied to the Calu-3 cells. It was, however, not investigated how the OVA:CAF01 complex might interact with the mucus with subsequent increased permeability of OVA through the mucus and retention in the epithelium.

Another concern associated with vaccination via the airways is the risk for damage to the epithelial cells. This was addressed *in vitro* by investigating the effect of CAF01 on the Calu-3 cell model measuring TEER to study the integrity of the epithelium and the formation of formazan from MTS to study the mitochondrial activity, and thereby the viability of the cells post exposure to ^{14}C-OVA in the presence or absence of CAF01. No changes in epithelial integrity and viability of the epithelial cells were observed, as compared to cells exposed to HBSS, which suggests that the epithelial cells were not affected by the application of the adjuvant system. This suggests that CAF01 is well-tolerated by the airway epithelium. Previous studies regarding the epithelial toxicity of DDA liposomes (not containing TDB) in mice supported this since DDA did not alter the macroscopic histology of the nasal epithelium after up to 60 min exposure (Klinguer et al., 2001). This, however, has to be investigated further with, e.g. histopathological investigations of the nasal cavity and lungs after *in vivo* administration of the CAF01 vaccine.

The present study demonstrates that intranasal administration of the influenza split vaccine adjuvated with CAF01 can induce significantly stronger immune responses in BALB/c mice compared to administration without adjuvant. Both cellular and humoral immune responses were increased, inducing both significantly higher IFN-γ and IgG levels, respectively. It would be relevant to compare this route of administration with more conventional routes, like s.c. and i.m. vaccination, in which cases one might expect a higher systemic response than with *i.n.* vaccination, where mucosal immunity, e.g. IgA would be more prevalent. The applied administration procedure, however, is suboptimal, since a droplet of the vaccine is placed in the nostrils of the mice anaesthetised transiently with CO_2. This implies a substantial risk that the mice will swallow the vaccine or sneeze it out, when they wake up. Furthermore, studies have shown that delivery to the lower respiratory tract would increase the efficacy of the vaccine as compared to administration to the nasal cavity (like in the present experiment) and upper respiratory tract (Smith et al., 2003; Minne et al., 2007). There is therefore reason to believe that further optimization of the application method will increase the immune response even further. One possible method could be to use a pulmonary application method like the one described using a MicroSprayer™ aerosolizer intended for endotracheal administration in mice (Bivas-Benita et al., 2005). In ongoing studies, this method is being investigated and the nasal and pulmonary routes of administration are compared with the more conventional s.c. and i.m. routes. At the same time the systemic and mucosal immune responses are analysed in more detail, determining IgA levels in the lungs, iso-typing serum IgG and investigating memory capacity of the differentiating T-cells, using triple icFACS to characterize the IFN-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-α producing T-cell populations.

5. Conclusion

CAF01 is a promising adjuvant for mucosal vaccination, significantly increasing the amount of antigen permeating the viscous mucus barrier and penetrating into the epithelium *in vitro*. CAF01 does not compromise the integrity and viability of the epithelial cells. Further, in a murine *in vivo* model, it induced significantly stronger immune responses towards an influenza vaccine.

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