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# Prevention of influenza virus infection and transmission by intranasal administration of a porous maltodextrin nanoparticle-formulated vaccine

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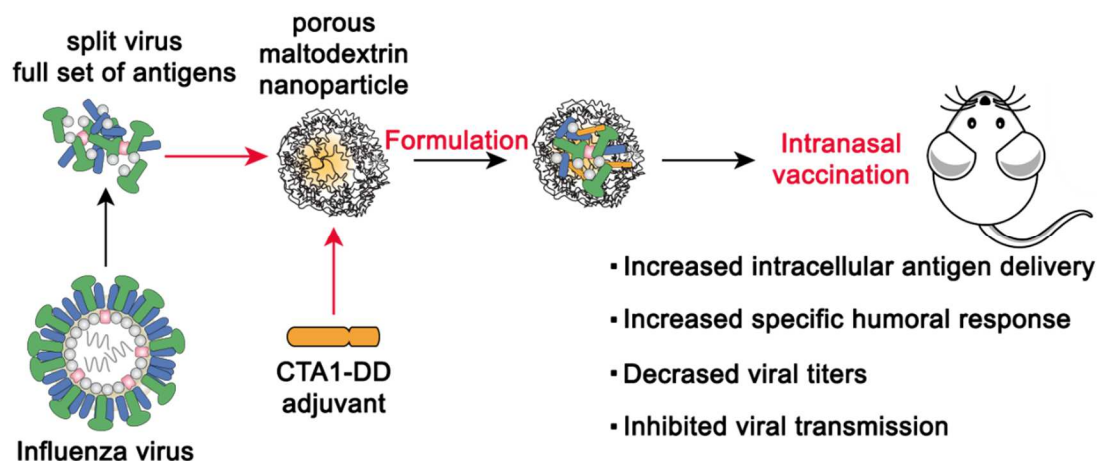
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## ABSTRACT

Influenza vaccines administered intramuscularly exhibit poor mucosal immune responses in the respiratory tract which is the prime site of the infection. Intranasal vaccination is a potential route for vaccine delivery which has been demonstrated effective in inducing protective immune responses in both systemic and mucosal compartments. For this purpose, nanoparticles have been used as antigen delivery systems to improve antigen capture by immune cells. In this paper we demonstrate efficient delivery of viral antigens to airway epithelial cells, macrophages and dendritic cells, using polysaccharide nanoparticles (NPL), leading to a strong protection against influenza virus infection. A formulation combining split Udm virus antigens with NPL and the mucosal protein adjuvant CTA1-DD was administered intranasally and resulted in an enhanced specific humoral immune response. Furthermore, NPL carrying split Udm, with or without CTA1-DD, inhibited virus transmission from infected to uninfected naive mice. These results demonstrate that an intranasal delivery system combining NPL, mucosal adjuvant CTA1-DD and split virus antigens confers robust protection against influenza infection and inhibits virus transmission.



## Graphical ABSTRACT

## 1. INTRODUCTION

Influenza is a highly-contagious respiratory infectious disease which causes public health and socio-economic problems worldwide (Gasparini et al., 2012). Many studies have focused on the development of vaccines against the Influenza virus, and this has led to the commercialization of 'flu' vaccines over the last fifty years (Yusuf and Kett, 2017). Among the ongoing vaccination strategies, the intranasal route has become very promising: over and above the fact that it is a needle-free and painless method, intranasal vaccination has been found superior to systemic immunizations for stimulating strong mucosal IgA and resident memory T cell responses and further results in systemic activation of the immune system via the nasal associated lymphoid tissue (NALT). This tissue, located inside and underneath the nasal epithelium, contains epithelial microfold cells (M cells) that overlay antigen presenting cells (APC) such as dendritic cells (DCs), macrophages and immature B cells (Davis, 2001). The M cells are able to transcytose antigens from the environment to the underlying APC, and mature APC will present antigen to naïve T cells in the draining lymph nodes. While B cell activation will lead to plasma cell development and antibody production, T cell activation will generate CD4 and/or CD8 T cells against the invading pathogen. Both humoral and cellular responses can occur locally at the administration site, but also throughout the whole body via a colonization of the spleen and lymph nodes (Brandtzaeg, 2009; Neutra and Kozlowski, 2006).

Currently available influenza vaccines are either live, attenuated viruses or subunit/split viral antigens (Tregoning et al., 2018). The attenuated vaccines are highly immunogenic but are often unstable and can cause adverse reactions, particularly in immunocompromised individuals (Bernstein et al., 1982), while subunit/split vaccines confer only limited immunogenicity (Grohskopf et al., 2018; Neuzil et al., 2006). It is nevertheless possible to

enhance this immunogenicity, while avoiding high antigen doses, through the use of immune modulators such as adjuvants or vaccine delivery systems.

The cholera toxin (CT) is one of the most common adjuvants used in veterinary vaccines (Boyaka, 2017). It binds ganglioside GM1-receptors present on APC, and when administered with antigens it augments antigen-specific mucosal sIgA and systemic IgG productions (Cox et al., 2006; Giri and Khuller, 2008). Owing to its toxicity, however, CT cannot be used as an adjuvant in human vaccines (Akhiani et al., 2006). Consequently, a non-toxic derivative of CT was developed, where the CTA1 subunit is expressed in a fusion protein together with a dimer of the D-fragment of proteinA from *Staphylococcus aureus* bacteria (DD) (Agren et al., 1997). Candidate vaccines containing CTA1-DD have been shown to induce strong mucosal responses (Lycke and Lebrero-Fernandez, 2018) and to enhance immunity to HIV-1, rotavirus, Influenza A virus and other pathogens, confirming its adjuvant properties (Sundling et al., 2008).

Finally, nanodelivery systems made from mucoadhesive polymers such as starch, chitosan or hyaluronic acid offer significant potential for enhancing the immunogenicity of mucosally-administered antigens (Cordeiro et al., 2015; Sharma et al., 2015). Such carriers can prolong the antigen residence time in the nasal mucosa, protect the antigens against proteolysis and specifically target APC. Porous and cationic maltodextrin-based nanoparticles with a phospholipid core (NPL) have received particular attention for the delivery of antigens via the nasal route. Previous studies showed that NPL were able to be loaded with large amounts of proteins, and that their residence time in the first layer of the nasal mucosa after administration was longer than antigens alone. Moreover, NPL have already been safely and successfully used as a nanocarrier for mucosal vaccines (Bernocchi et al., 2016; Carpentier et al., 2018; Dimier-Poisson et al., 2015; Ducournau et al., 2017).

Most vaccines against influenza infection are generally administered intramuscularly using inactivated influenza virus antigens (IIV). They are painful, sometimes adjuvant-supplemented and not fully effective (Poehling et al., 2018). As an alternative, the intranasally administered Flumist® vaccine, a live attenuated influenza vaccine (LAIV), underlines the potential of mimicking a natural influenza infection able to trigger protective mucosal immune response. However, the inconsistent effectiveness of nasal LAIV coupled with production and storage issues have resulted in the withdrawal of recommendations for its use in the United States (Furlow, 2017). Moreover, the American Center for Disease Control and prevention (CDC) recently reported an overall effectiveness of flu vaccines of only around 45% for the 2019-2020 season (<https://www.cdc.gov/flu/vaccines-work/2019-2020.html>), meaning there is an important gap to be filled in terms of flu vaccine efficiency.

To combine the advantages of both the IIV and LAIV-Flumist® approaches, and to increase effectiveness, we previously developed an influenza vaccine comprising a chimeric fusion protein (CTA1-M2e(3x)-DD) associated with NPL (Bernasconi et al., 2018) and showed that this combination decreased both the virus's mortality and its transmission. However, we did not demonstrate complete protection of this vaccine against infection.

Consequently, to increase the protection offered by our nanoparticle-assisted influenza nasal vaccine, in this study we used the full set of flu antigens (including seasonal and conserved antigens). We evaluated the immunogenicity of an intranasal vaccine against the influenza virus, comprised of split Udorn virus admixed or not with CTA1-DD adjuvant (Lycke, 2012) and encapsulated in NPL. The viral antigen loading in NPL and the delivery studies were first performed *in vitro* in nasal mucosal cells. Immune protection was then examined *in vivo* in intranasally immunized mice by measuring serum Udorn-specific IgG titers, and the viral titers in the snouts and lungs after a nasal infection with influenza. Finally, the transmission of

infectious influenza virus was examined by measuring the viral titers in unvaccinated mice co-housed with vaccinated and infected animals.

## **2. MATERIALS AND METHODS**

### **2.1. Animals**

Six- to eight-week-old BALB/c and DBA/2J mice were purchased from Janvier Laboratories (Strasbourg, France). Mice were kept under specific-pathogen-free conditions in the local animal facility. All experiments were approved by the local animal welfare committee and were performed in accordance with the guidelines of the Federation for Laboratory Animal Science Associations (FELASA), the national animal welfare body, and complied with the German animal protection law.

### **2.2. NPL synthesis**

The NPL were prepared as described previously (Le et al., 2018; Paillard et al., 2010). Maltodextrin was dissolved in 2 N sodium hydroxide with magnetic stirring at room temperature and epichlorhydrin and glycidyltrimethylammonium chloride were added to make a cationic polysaccharide gel. The gel was then neutralized with acetic acid and sheared with a high-pressure homogenizer (LM 20, Microfluidics, France). The particles thus obtained were purified by tangential flow ultra-filtration (AKTA flux 6, GE Healthcare, France) using a 750 kDa membrane (GE Healthcare, France) and mixed with 70% 1,2-Dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) (weight %) above the gel-to-liquid phase transition temperature to produce NPL.

### **2.3. Virus production, inactivation and labeling**

Udorn virus (Influenza A virus A/Udorn/307/72, H3N2) strain was amplified using MDCK (Madin-Darby Canine Kidney) cells in serum-free Dulbecco's Modified Eagle medium (DMEM), supplemented with non-essential amino acids, 2 mM L-glutamine and 0.4 mM sodium pyruvate, at 37 °C in 5% CO<sub>2</sub>. Ninety six hours after virus inoculation, the culture medium was collected by centrifugation for 10 min at 2500 g and 4 °C, and the virus was pelleted from the supernatants by overnight centrifugation at 30,000 g and 4 °C. Viral titers were determined by plaque formation on MDCK cells (Kolpe et al., 2018).

Udorn virus was inactivated following incubating with  $\beta$ -propiolactone ( $\beta$ -BPL) for 16 hours at 4 °C, then subsequently incubated at 37 °C for 2 hours to facilitate hydrolysis of  $\beta$ -BPL. Inactivated virus were stored at -80 °C. Triton X-100 was added at a final concentration of 10% (v:v) to split the Udorn virus.

The split Udorn virus was then covalently labeled with 0.7% FITC (Fluorescein isothiocyanate, weight %) in 0.1 M bicarbonate buffer (pH 8.3), and the solution was mixed for 6 h in the dark at room temperature. The labeled virus was purified by gel filtration on a PD-10 Sephadex column and the concentration of the labeled protein was then evaluated using the Micro BCA Protein Assay Kit following the supplier's instructions (ThermoFisher Scientific, France).

#### **2.4. Protein loading into nanoparticles**

CTA1-DD mucosal adjuvant has been described previously (Bernasconi et al., 2018). Based on their protein concentrations, CTA1-DD and split Udorn virus were loaded into sterile NPL by mixing both components at room temperature obtaining 1:5 split Udorn:NPL or 1:1:5 split Udorn:CTA1-DD:NPL (mass ratios) vaccine formulations.

#### **2.5. Characterization of the nanoparticle and formulations**



The hydrodynamic diameter (Z-average) of nanoparticles was measured by dynamic light scattering in 23 mM NaCl. The zeta potential was measured by electrophoretic mobility in pure water. Measurements were carried out in triplicate using a Zetasizer nanoZS (Malvern Instruments, France) with a NPL concentration of 100 µg/ml.

## **2.6. Characterization of the association of proteins and split virus with NPL**

The protein association of CTA1-DD and the split Udorn virus with NPL was evaluated using native polyacrylamide gel electrophoresis (native PAGE). Formulations were supplemented with a non-denaturing buffer (125 mM Tris-HCl (pH 6.8), 10% glycerol and 0.06% bromophenol blue) and run on a 10% acrylamide-bisacrylamide gels stained by the silver nitrate method. Gels were scanned and quantify using the ImageJ software. Under these conditions, non-associated proteins entered the gel while NP-associated proteins did not.

## **2.7. Intracellular delivery of split Udorn by NPL**

The H292 (NCI-H292, ATCC #CRL-1848) and PMA-differentiated (Le et al., 2018) THP-1 (ATCC #TIB-202) cells were maintained in RPMI supplemented with 10 % heat-inactivated, fetal calf serum (FCS), 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine at 37 °C in an humidified, 5% CO<sub>2</sub> atmosphere. The SRDC (Le et al., 2018; Ruiz et al., 2005) were cultured in IMDM supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine at 37 °C in an humidified, 5% CO<sub>2</sub> atmosphere. Cells were plated at a density of 5x10<sup>5</sup> cells for 3 days in 6-well plates and treated for 3 h with 1 µg of split Udorn-FITC either free or loaded into nanoparticles. The cells were then collected by trypsinization and analyzed by flow cytometry using an Accuri™ C6 CFlow Sampler flow cytometer (BD Bioscience, USA). **The cells were gated using the SSC/FSC parameters and the FL1 channel (laser 488nm, filter 533/30nm) was used to analyze**

the split Udorn-FITC intracellular delivery. Untreated cells were used as an auto-fluorescence negative control.

## **2.8. Vaccination of mice**

Six- to eight-week-old female BALB/c mice were immunized three times by the intranasal route (i.n.), 10 days apart, with 10  $\mu$ l (5  $\mu$ l per nostril) containing either (a) 1  $\mu$ g of split Udorn alone, (b) 1  $\mu$ g of split Udorn formulated with 5  $\mu$ g of NPL or (c) 1  $\mu$ g of split Udorn and 1  $\mu$ g of CTA1-DD formulated with 5  $\mu$ g of NPL. Ten days after the first and the second immunizations, serum samples were collected in order to measure the levels of anti-HA specific antibodies by ELISA.

## **2.9. Detection of anti-Udorn antibodies in serum**

To determine Udorn-specific antibody titers, microtiter plates (MaxiSorp, Nunc) were coated with split Udorn virus, and incubated overnight at 4 °C. After washing, the plates were blocked for 2 h with 5% BSA in PBS. After blocking, a series of 1/3 dilutions of the different serum samples were loaded on the peptide-coated plates. The bound antibodies were measured with a horseradish peroxidase-labeled antibody directed against mouse IgG, diluted 1/2000 (Thermo Fisher Scientific, USA). After washing, the microtiter plates were incubated for 20 min with a TMB substrate (Tetramethylbenzidine, Life technologies). The reaction was stopped by adding 0.5 M H<sub>2</sub>SO<sub>4</sub> and absorbance was measured at 450 nm. The endpoint titers were defined as the highest dilution resulting in an O.D. value twice that of the background (pre-immune serum).

## **2.10. Virus transmission study in naive mice**

Six- to eight-week-old female BALB/c mice were immunized three times (10 days apart) by the intranasal route with either (a) 1  $\mu$ g of split Udorn alone, (b) 1  $\mu$ g of split Udorn

formulated with 5 µg of NPL, (c) 1 µg of split Udorn and 1 µg of CTA1-DD formulated with 5 µg of NPL, or (d) with a mock vaccine composed of 1 µg of ovalbumin (OVA) loaded into 5 µg of NPL. Five weeks after the final immunization, all immunized BALB/c mice were infected intranasally with 20 µl containing  $3 \times 10^4$  PFU H3N2 Udorn virus (A/Udorn/307/1972 – H3N2) and were used as index animals. At 24 h post-infection, these index mice were co-housed one-to-one with uninfected, 6-week-old DBA/2 mice as contact mice in a clean cage. The snouts and the lungs of the index and contact mice were collected after 4 days post co-habitation and viral loads were determined by viral titration assay.

### **2.11. Viral titration**

The MDCK cells were maintained in DMEM supplemented with 10% FCS, non-essential amino acids, 2 mM L-glutamine and 0.4 mM sodium pyruvate and maintained at 37 °C in a 5% CO<sub>2</sub> atmosphere. Cells were grown to a confluent layer in 12-well plates for the titration assay. Snouts and lungs were homogenized in 800 µl cold PBS using FastPrep® spheres and tubes in an homogenizer (3 cycles at 6.5 m/s for 20 seconds). Samples were then centrifuged for 10 min at 9,000 rpm and 4 °C, and supernatants were diluted 10-fold in OptiMEM (Thermo Fisher Scientific) supplemented with 0.3% BSA. A volume of 450 µl of these diluted samples were added to confluent MDCK cells for 2 h at room temperature. After removing the inoculum, cells were overlaid for 72 h with 1.5% Avicel (FMC BioPolymer) in DMEM supplemented with 10% BSA. Cells were then fixed with 3.7% formaldehyde and stained with 1% crystal violet solution for 15 min. Plaques were used to calculate virus titers in plaque-forming units (pfu) per ml.

### **2.12. Statistics**

Data were analyzed by one-way and two-way ANOVA using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA).

### 3. RESULTS

#### 3.1. Characterization of NPL and formulations

The NPL had a hydrodynamic diameter of 38 nm while the average size of the split Udorn was 111 nm. Moreover NPL possessed a positive surface charge (+38 mV) while split Udorn virus had a negative charged (-20 mV). The association of the split Udorn with NPL at a mass ratio of 1:5 (protein:NPL) yielded formulation of the same size as NPL alone (40 nm), with a positive surface charge (+26 mV) which implied that split Udorn antigens were successfully loaded inside the NPL (Fig 1).

Similarly, the association between split Udorn, CTA1-DD and NPL (mass ratio 1:1:5) showed no increase of the size of NPL and a zeta potential of +26 mV, again confirming the encapsulation (Fig 1).

In addition to size and zeta potential analysis, the loading of proteins into NPL was assessed by native PAGE (Fig 2). Splitting the virus did not modify the protein pattern of the Udorn virus. The split Udorn was completely encapsulated by NPL as no free protein was observed, while the combination of split Udorn and CTA1-DD with NPL resulted in a loading efficacy of 90% of split Udorn. Taken together, these data added further evidence for the encapsulation of the majority of Udorn antigens and the mucosal adjuvant by NPL at mass ratio 1:1:5 (Fig 2 and Table 1).

Formulations	Percentage of split Udorn loading
Split Udorn/NPL	100%
Split Udorn/CTA1-DD/NPL	90%

**Table 1: Loading efficacy of split Udorn and split Udorn/CTA1-DD in NPL.** Non-denaturing PAGE (from figure 2) were analyzed by densitometry using ImageJ. The percentages of association with the NPL were calculated over the 100% Split Udorn input and reported in the table.

### **3.2. Cellular delivery of split Udorn by NPL**

We investigated the delivery of split Udorn by NPL into 3 different airway mucosa cell lines. Using fluorescently-labeled Udorn viral proteins, flow cytometry analysis showed that NPL significantly increased the uptake of split Udorn antigens by H292 airway epithelial cells, SRDC dendritic cells and THP-1 macrophages. The delivery was 4 times higher in H292 and SRDC, and 5 times higher in THP-1 macrophages (Fig 3).

### **3.3. Udorn-specific antibody titers after intranasal vaccination**

To investigate the efficacy of NPL-formulated vaccines, we compared the serum antibodies in mice after intranasal vaccination. As a negative control, mice were vaccinated with OVA/NPL, otherwise mice were immunized intranasally with split Udorn alone, split Udorn/NPL, or split Udorn/CTA1-DD/NPL. The levels of Udorn-specific serum IgG of vaccinated mice are shown in Figure 4. Vaccination with all of the above formulations increased Udorn-specific IgG antibody production, and it was enhanced by a second immunization. After the second immunization, a significant increase of Udorn-specific IgG was induced by split Udorn/NPL compared with split Udorn alone. Moreover, adding the CTA1-DD adjuvant to the split Udorn/NPL formulation induced a greater increase of the specific serum immune response, though not significantly different from the split Udorn/NPL formulation. Altogether, these results reveal that both NPL and CTA1-DD have immune-enhancing effects after intranasal administration.

### **3.4. Protection against virus transmission**

We then determined whether intranasal administration of NPL-encapsulated, CTA1-DD-adjuvanted, split Udorn vaccine was sufficient to induce mucosal immunity and protection against influenza virus infection. Two weeks after final vaccination, we challenged immunized mice (referred to as index mice) intranasally with 20  $\mu$ l containing  $3 \times 10^4$  PFU/animal of H3N2 Udorn virus and co-housed them 24 hours later with naïve, non-immunized, contact mice. Virus transmission was assessed by monitoring the snouts and lungs for viral titers of both index and contact mice after 4 days of one-to-one co-habitation.

Index mice immunized with split Udorn/NPL exhibited significantly lower viral titers in the snout compared with non-immunized mice (OVA/NPL group) and this was further enhanced with the split Udorn/CTA1-DD/NPL formulation. In the lungs of index mice, only the immunization with intranasal split Udorn/CTA1-DD/NPL significantly reduced the Udorn viral titers (Fig 5a).

Though index mice vaccinated with split Udorn alone were poorly protected from virus infection (Fig 5a), they showed a limited virus transmission (25% of mice compared to 100% transmission using OVA/NPL immunization). Importantly, index mice vaccinated with split Udorn/NPL formulations, with or without CTA1-DD, did not transmit the influenza virus to contact mice (100% of virus transmission prevention, Fig 5b). This demonstrates that NPL were effective in limiting virus transmission.

#### **4. DISCUSSION**

NPL are cationic maltodextrin nanoparticles with a phospholipid core. They are highly and rapidly endocytosed by airway epithelial cells, dendritic cells (DCs) and macrophages (Le et al., 2018) and significantly improve protein delivery into those cells, even at low doses (Bernocchi et al., 2016; Le et al., 2019). In addition, vaccination studies against toxoplasmosis

infection have shown that NPL-based vaccines protect animals against chronic and congenital infection, confirming their efficiency as vaccine delivery systems (Dimier-Poisson et al., 2015; Ducournau et al., 2017). Moreover CTA1-DD has been used as an adjuvant for mucosal vaccines (Lycke and Lebrero-Fernandez, 2018; Sundling et al., 2008) and it was originally developed to circumvent the toxicity of CT holotoxin; it was found to augment mucosal and systemic immune responses comparable to that of CT, but without toxic side effects (Dhere et al., 2011; Lycke, 2012). Hence this study aimed to evaluate the effect of NPL as a delivery system of vaccine antigens and mucosal adjuvants.

The positive surface charge and lipid core of NPL facilitates both electrostatic and hydrophobic interactions between the nanocarrier and antigens. Moreover, their porous structure allows NPL to be loaded with high amount of antigens (Dimier-Poisson et al., 2015; Le et al., 2019). Split Udorn virus and the CTA1-DD adjuvant used in this study were shown to be >90% loaded into the NPL by Native PAGE assays (Fig 1 and 2, Table 1). Importantly, NPL very efficiently delivered split Udorn into 3 main cell types present in the nasal mucosa. The improvement in uptake (Fig 3) and processing (Bernasconi et al., 2018) of the associated Udorn antigens by antigen-presenting cells make NPL an ideal nanodelivery system for intranasal vaccines.

Many examples have shown that using an adjuvant in combination to a nanodelivery system is sometimes necessary to create significant protection against the influenza virus. It was demonstrated that recombinant HA nanoparticle-based influenza vaccine required a saponin-based adjuvant to induce protection against influenza A(H3N2) strains (Smith et al., 2017). Furthermore PEG-liposomes containing CRX-601 adjuvant and methylglycol chitosan also increased the murine sublingual immune response to influenza vaccination (Oberoi et al., 2016). Here, the *in vivo* study showed that intranasal administration of a split Udorn/NPL vaccine enhances Udorn-specific IgG antibody response as compared to mice receiving split

Udorn alone. Moreover, adding CTA1-DD into the split Udorn/NPL formulation tended to improve the level of Udorn-specific IgG compared with administration of split Udorn alone (Fig 4), but not significantly more so than the split Udorn/NPL formulation. This is in agreement with our recently published results (Bernasconi et al., 2018) and suggests that, combined with split Udorn antigens, an efficient delivery system (NPL) is sufficiently effective even in the absence of adjuvant. This pattern was also seen in the transmission study we performed (Fig 5).

Noteworthy, influenza virus protection should be assessed in the snout and lungs as these are the entry point for infection (Kohlmeier and Woodland, 2009). We therefore analyzed the protection induced by NPL-formulated influenza vaccine in snouts and lungs of infected and immunized mice. Correlated with the production of Udorn-specific IgG (Fig 4), the NPL appears essential to limit both viral infection and transmission, an effect enhanced by the CTA1-DD adjuvant in the case of Udorn antigens compared with vaccination using the split virus alone (Fig 5). In addition, mucosal immunity is a key objective when seeking to limit influenza infection and transmission. We have previously demonstrated that NPL, associated with a chimeric antigen of influenza virus, induced the mucosal secretion of specific IgA while antigen alone could not (Bernasconi et al., 2018). Here, our strategy was designed to mimic a natural infection and to deliver antigens into mucosa cells. Further experiments will be performed to characterize the mucosal immunity triggered by our NPL-based vaccine, especially measuring humoral response and T-cell activation.

In these first experiments using NPL associating the total split Udorn virus, we immunized mice three times while human vaccinations led by public health authorities are often single-dose (IIV or LAIV). However, experimental mice are naïve to flu virus infection and booster administrations are required, contrary to humans that have been previously infected by flu viruses having shared conserved epitopes.



We conclude that the use of an adjuvant in combination with a nanocarrier did not appear to be an absolute necessity for conferring 100% protection against influenza infection which agrees with a NPL-assisted anti-*Toxoplasma gondii* vaccine (Dimier-Poisson et al., 2015; Ducournau et al., 2017) that does not require the use of any additional adjuvant. Finally, this study emphasizes the interest of using NPL as a vaccine delivery system for influenza antigens and adjuvant.

## **5. CONCLUSION**

Intranasal administration of a split Udorn virus using an NPL nanocarrier, with or without the CTA1-DD adjuvant, is an efficient vaccine strategy for stimulating immunity against influenza virus. Importantly, the mice vaccinated with NPL formulations were not only protected against infection, but did also not transmit the influenza infection, an often overlooked parameter of immune protection.

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## **CONFLICT OF INTEREST**

The authors declare no conflict of interest.

## REFERENCES

- Agren, L.C., Ekman, L., Lowenadler, B., Lycke, N.Y., 1997. Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit. *J Immunol* 158, 3936-3946.
- Akhiani, A.A., Stensson, A., Schon, K., Lycke, N., 2006. The nontoxic CTA1-DD adjuvant enhances protective immunity against *Helicobacter pylori* infection following mucosal immunization. *Scand J Immunol* 63, 97-105.
- Bernasconi, V., Bernocchi, B., Ye, L., Le, M.Q., Omokanye, A., Carpentier, R., Schon, K., Saelens, X., Staeheli, P., Betbeder, D., Lycke, N., 2018. Porous Nanoparticles With Self-Adjuvanting M2e-Fusion Protein and Recombinant Hemagglutinin Provide Strong and Broadly Protective Immunity Against Influenza Virus Infections. *Front Immunol* 9, 2060.
- Bernocchi, B., Carpentier, R., Lantier, I., Ducournau, C., Dimier-Poisson, I., Betbeder, D., 2016. Mechanisms allowing protein delivery in nasal mucosa using NPL nanoparticles. *J Control Release* 232, 42-50.
- Bernstein, D.I., Zahradnik, J.M., DeAngelis, C.J., Cherry, J.D., 1982. Clinical reactions and serologic responses after vaccination with whole-virus or split-virus influenza vaccines in children aged 6 to 36 months. *Pediatrics* 69, 404-408.
- Boyaka, P.N., 2017. Inducing Mucosal IgA: A Challenge for Vaccine Adjuvants and Delivery Systems. *J Immunol* 199, 9-16.
- Brandtzaeg, P., 2009. Mucosal immunity: induction, dissemination, and effector functions. *Scand J Immunol* 70, 505-515.
- Carpentier, R., Platel, A., Salah, N., Nesslany, F., Betbeder, D., 2018. Porous Maltodextrin-Based Nanoparticles: A Safe Delivery System for Nasal Vaccines. *Journal of Nanomaterials*.
- Cordeiro, A.S., Alonso, M.J., de la Fuente, M., 2015. Nanoengineering of vaccines using natural polysaccharides. *Biotechnol Adv* 33, 1279-1293.
- Cox, E., Verdonck, F., Vanrompay, D., Goddeeris, B., 2006. Adjuvants modulating mucosal immune responses or directing systemic responses towards the mucosa. *Vet Res* 37, 511-539.
- Davis, S.S., 2001. Nasal vaccines. *Adv Drug Deliv Rev* 51, 21-42.
- Dhere, R., Yeolekar, L., Kulkarni, P., Menon, R., Vaidya, V., Ganguly, M., Tyagi, P., Barde, P., Jadhav, S., 2011. A pandemic influenza vaccine in India: from strain to sale within 12 months. *Vaccine* 29 Suppl 1, A16-21.
- Dimier-Poisson, I., Carpentier, R., N'Guyen, T.T., Dahmani, F., Ducournau, C., Betbeder, D., 2015. Porous nanoparticles as delivery system of complex antigens for an effective vaccine against acute and chronic *Toxoplasma gondii* infection. *Biomaterials* 50, 164-175.
- Ducournau, C., Nguyen, T.T., Carpentier, R., Lantier, I., Germon, S., Precausta, F., Pisella, P.J., Leroux, H., Van Langendonck, N., Betbeder, D., Dimier-Poisson, I., 2017. Synthetic parasites: a successful mucosal nanoparticle vaccine against *Toxoplasma congenital* infection in mice. *Future Microbiol* 12, 393-405.
- Furlow, B., 2017. US authorities discourage use of nasal influenza vaccine, citing ineffectiveness. *The Lancet. Respiratory medicine* 5, 105-106.
- Gasparini, R., Amicizia, D., Lai, P.L., Panatto, D., 2012. Clinical and socioeconomic impact of seasonal and pandemic influenza in adults and the elderly. *Hum Vaccin Immunother* 8, 21-28.
- Giri, P.K., Khuller, G.K., 2008. Is intranasal vaccination a feasible solution for tuberculosis? *Expert Rev Vaccines* 7, 1341-1356.
- Grohskopf, L.A., Sokolow, L.Z., Broder, K.R., Walter, E.B., Fry, A.M., Jernigan, D.B., 2018. Prevention and Control of Seasonal Influenza with Vaccines: Recommendations of the Advisory Committee on Immunization Practices-United States, 2018-19 Influenza Season. *MMWR Recomm Rep* 67, 1-20.
- Kohlmeier, J.E., Woodland, D.L., 2009. Immunity to respiratory viruses. *Annu Rev Immunol* 27, 61-82.
- Kolpe, A., Schepens, B., Ye, L., Staeheli, P., Saelens, X., 2018. Passively transferred M2e-specific monoclonal antibody reduces influenza A virus transmission in mice. *Antiviral Res* 158, 244-254.
- Le, M.Q., Carpentier, R., Lantier, I., Ducournau, C., Dimier-Poisson, I., Betbeder, D., 2018. Residence time and uptake of porous and cationic maltodextrin-based nanoparticles in the nasal mucosa: Comparison with anionic and cationic nanoparticles. *Int J Pharm* 550, 316-324.
- Le, M.Q., Carpentier, R., Lantier, I., Ducournau, C., Fasquelle, F., Dimier-Poisson, I., Betbeder, D., 2019. Protein delivery by porous cationic maltodextrin-based nanoparticles into nasal mucosal cells: Comparison with cationic or anionic nanoparticles. *International Journal of Pharmaceutics: X* 1, 100001.
- Lycke, N., 2012. Recent progress in mucosal vaccine development: potential and limitations. *Nat Rev Immunol* 12, 592-605.
- Lycke, N., Lebrero-Fernandez, C., 2018. ADP-ribosylating enterotoxins as vaccine adjuvants. *Curr Opin Pharmacol* 41, 42-51.
- Neutra, M.R., Kozlowski, P.A., 2006. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol* 6, 148-158.

Neuzil, K.M., Jackson, L.A., Nelson, J., Klimov, A., Cox, N., Bridges, C.B., Dunn, J., DeStefano, F., Shay, D., 2006. Immunogenicity and reactogenicity of 1 versus 2 doses of trivalent inactivated influenza vaccine in vaccine-naïve 5-8-year-old children. *J Infect Dis* 194, 1032-1039.

Oberoi, H.S., Yorgensen, Y.M., Morasse, A., Evans, J.T., Burkhart, D.J., 2016. PEG modified liposomes containing CRX-601 adjuvant in combination with methylglycol chitosan enhance the murine sublingual immune response to influenza vaccination. *J Control Release* 223, 64-74.

Paillard, A., Passirani, C., Saulnier, P., Kroubi, M., Garcion, E., Benoit, J.P., Betbeder, D., 2010. Positively-charged, porous, polysaccharide nanoparticles loaded with anionic molecules behave as 'stealth' cationic nanocarriers. *Pharm Res* 27, 126-133.

Poehling, K.A., Caspard, H., Peters, T.R., Belongia, E.A., Congeni, B., Gaglani, M., Griffin, M.R., Irving, S.A., Kavathekar, P.K., McLean, H.Q., Naleway, A.L., Ryan, K., Talbot, H.K., Ambrose, C.S., 2018. 2015-2016 Vaccine Effectiveness of Live Attenuated and Inactivated Influenza Vaccines in Children in the United States. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 66, 665-672.

Ruiz, S., Beauvillain, C., Mevelec, M.N., Roingard, P., Breton, P., Bout, D., Dimier-Poisson, I., 2005. A novel CD4-CD8alpha+CD205+CD11b- murine spleen dendritic cell line: establishment, characterization and functional analysis in a model of vaccination to toxoplasmosis. *Cell Microbiol* 7, 1659-1671.

Sharma, R., Agrawal, U., Mody, N., Vyas, S.P., 2015. Polymer nanotechnology based approaches in mucosal vaccine delivery: challenges and opportunities. *Biotechnol Adv* 33, 64-79.

Smith, G., Liu, Y., Flyer, D., Massare, M.J., Zhou, B., Patel, N., Ellingsworth, L., Lewis, M., Cummings, J.F., Glenn, G., 2017. Novel hemagglutinin nanoparticle influenza vaccine with Matrix-M adjuvant induces hemagglutination inhibition, neutralizing, and protective responses in ferrets against homologous and drifted A(H3N2) subtypes. *Vaccine* 35, 5366-5372.

Sundling, C., Schon, K., Morner, A., Forsell, M.N., Wyatt, R.T., Thorstensson, R., Karlsson Hedestam, G.B., Lycke, N.Y., 2008. CTA1-DD adjuvant promotes strong immunity against human immunodeficiency virus type 1 envelope glycoproteins following mucosal immunization. *J Gen Virol* 89, 2954-2964.

Tregoning, J.S., Russell, R.F., Kinnear, E., 2018. Adjuvanted influenza vaccines. *Hum Vaccin Immunother* 14, 550-564.

Yusuf, H., Kett, V., 2017. Current prospects and future challenges for nasal vaccine delivery. *Hum Vaccin Immunother* 13, 34-45.

## FIGURE CAPTIONS

**Figure 1: Size, PDI and zeta potential of NPL, split Udorn, CTA1-DD and the related formulations.** The hydrodynamic diameter (left), the polydispersity index -PDI- (middle) and the zeta potential (right) of NPL, split Udorn, CTA1-DD, split Udorn/NPL, split Udorn/CTA1-DD/NPL as measured by dynamic light scattering and electrophoretic mobility, respectively. The graphs represent the mean +/- SD of three independent measurements.

**Figure 2: Characterization of the split Udorn/NPL and split Udorn/CTA1-DD/NPL.** The encapsulation of antigens and adjuvant by NPL was characterized by non-denaturing polyacrylamide gel electrophoresis (PAGE) using formulations of 1:(1):5 (mass ratio of split Udorn:(CTA1-DD):NPL) ratios. Unbound proteins were revealed by silver nitrate staining.

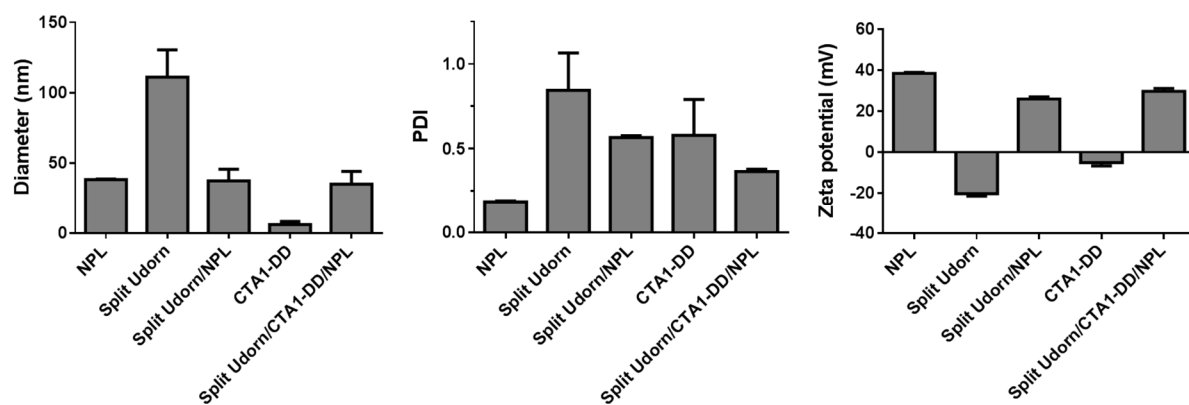
**Figure 3: Split Udorn delivery by NPL in H292 airway epithelial cells, SRDC dendritic cells and THP-1 macrophages.** Split Udorn was labeled with fluorescein and loaded or not into NPL at a mass ratio protein/NPL of 1:5. Cells were treated for 3 hours and the protein delivery was evaluated by flow cytometry. Statistical analysis: Two-way ANOVA; asterisks indicate p-values: \*\*\*  $p < 0.001$ .

**Figure 4: Serum Udorn-specific IgG titers from vaccinated mice.** Udorn-specific IgG titers were measured by ELISA in serum from mice immunized 3 times intranasally (a primary immunization followed by two boosters, 10 days apart) with OVA/NPL as negative control, split Udorn, split Udorn/NPL or split Udorn/CTA1-DD/NPL (mass ratio 1:(1):5). Statistical analysis: Two-way ANOVA. P-values: \*\*  $p < 0.01$  between indicated groups; \*\*\*\*  $p < 0.0001$  between indicated groups; \$  $p < 0.0001$  OVA/NPL vs. all other groups.

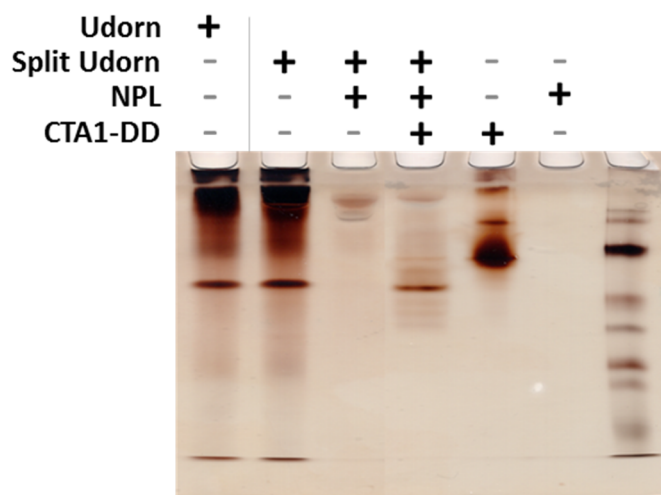
**Figure 5: Viral transmission from vaccinated mice.** Index mice were immunized with OVA/NPL as negative control, split Udorn, split Udorn/NPL or split Udorn/CTA1-DD/NPL (mass ratio 1:(1):5) and infected before being individually co-housed with non-vaccinated contact mice. A) The viral titers in index mice snouts and lungs; B) The viral titers in contact mice snouts. Statistical analysis: One-way or two-way ANOVA; asterisks indicate p-values:  $p < 0.0001$  (\*\*\*\*),  $p < 0.001$ ,  $p < 0.01$  (\*\*) and  $p < 0.05$  (\*).

**LOW RESOLUTION FIGURES FOR REVIEWING PURPOSE ONLY**

*High resolution figures are supplied in separated TIFF files*



**FIGURE 1**



**FIGURE 2**

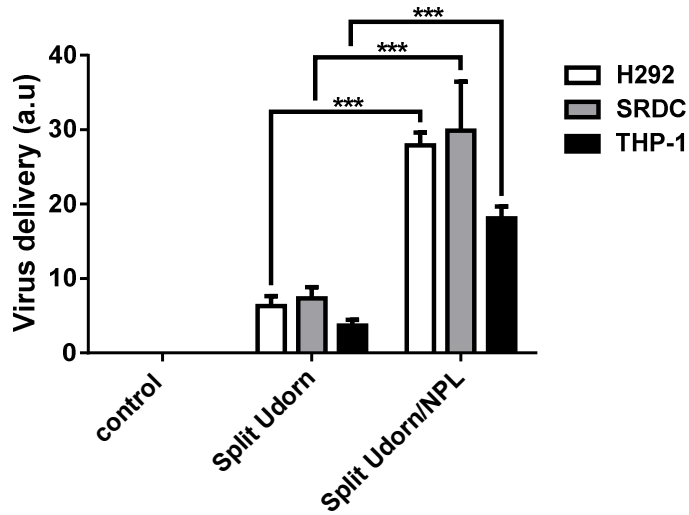


FIGURE 3

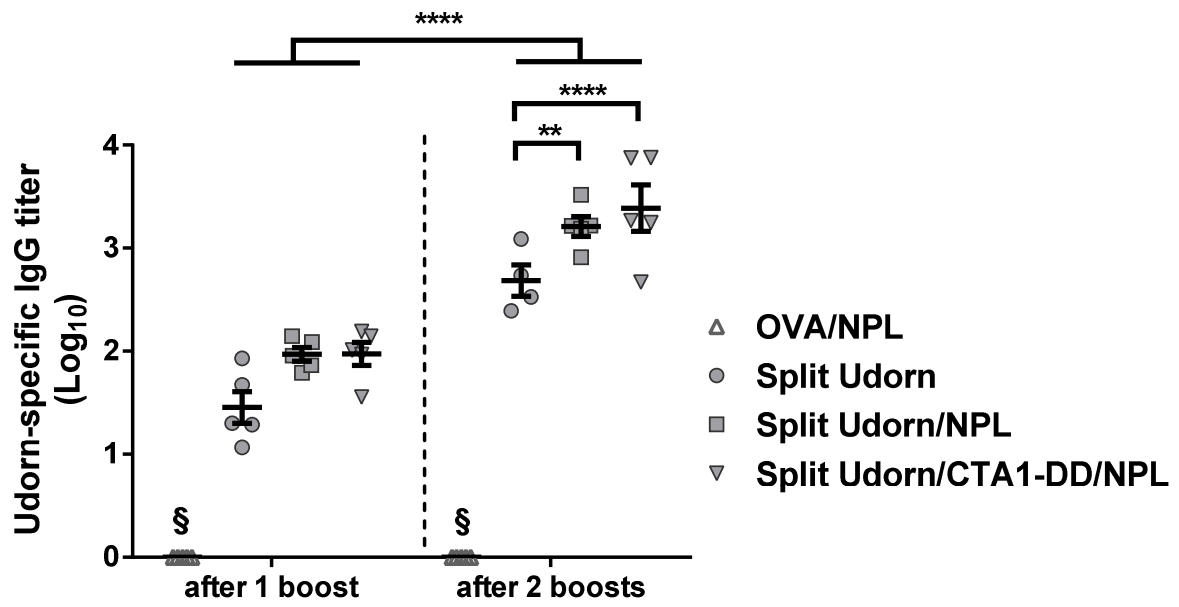
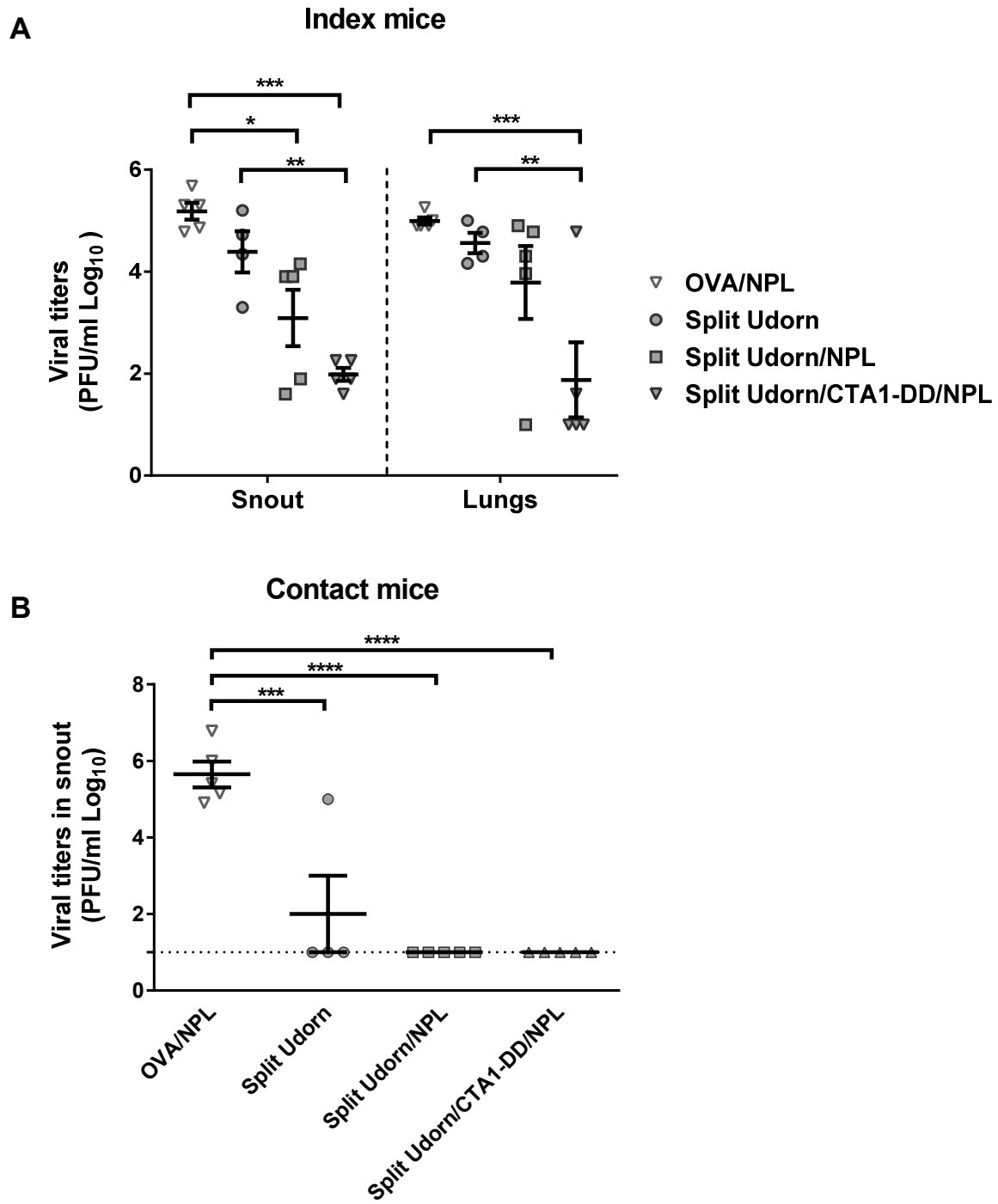


FIGURE 4



**FIGURE 5**