

# Adjuvanting of existing flu vaccines with Cationic Lipid DNA complexes (JuvImmune™) for cross-protection and dose sparing

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## INTRODUCTION

Influenza A infection causes substantial morbidity and mortality worldwide, particularly for infants, the elderly, and the immunocompromised. The efficacy of current vaccines is highly dependent on close matching of the hemagglutinin (HA) and neuraminidase (NA) surface proteins of the vaccine with currently circulating virus. Should neutralizing antibody fail to prevent infection of the respiratory tract, subsequent clearance of viral infection is mainly dependent on T cells, particularly cytolytic T lymphocytes (CTL) of the CD8<sup>+</sup> T-cell subset. Thus, it would be ideal for new adjuvants used with existing flu vaccines to induce both high levels of antibody and T-cell immunity. Cationic lipid DNA complexes (CLDC) is a unique adjuvant that is particularly promising for vaccines that induce both high levels of antibody and T-cell immunity, including CTL. The adjuvant is based on a cationic/neutral lipid carrier and non-coding DNA complex. Inclusion of protein antigens with CLDC results in an extremely robust humoral, CD4<sup>+</sup>, and CD8<sup>+</sup> immune response. The unique aspect of the CLDC adjuvant is that it functions both as a delivery system that targets associated antigens to antigen presenting cells via the liposome component and an immunostimulatory adjuvant via the plasmid DNA. In these studies the CLDC adjuvant was combined with whole inactivated virus or split vaccine (Fluzone® - Sanofi Pasteur) and administered to mice on day 0 and 14 and immune response and resistance to challenge monitored.

## MATERIALS AND METHODS

### Preparation of CLDC/Ag for SC dosing.

CLDC/Ag was prepared by the addition of 120µl of DOTIM/cholesterol liposomes to 590µl of 10% sucrose solution. To this mixture, 330µl antigen and 40µl of 3µg/µl plasmid DNA was added. The components were mixed by gentle pipetting prior to administration. For dose sparing studies the amount of antigen was decreased as indicated and the volume compensated for by additional 10% sucrose. Mice were administered 200µl of the test CLDC-antigen.

### Mouse immunization schedule.

Groups of 10 (CD1 or Balb/c) mice were immunized at days 0 and 14. Mice were sacrificed at 28 days to assess immunity and resistance to challenge.

### Anti-Flu antigen antibody titer ELISA.

Anti-flu antibody titer was determined by colorimetric ELISA. Antibody titer was defined as the midpoint of the dilution curve defined by EC<sub>50</sub> calculations using Prism statistical software. The average of the individual EC<sub>50</sub> measurements for each cohort was determined to be the antibody titer. Serum pooled from several mice that have been immunized twice with served as a positive control. This allowed normalization of experiments performed on different days.

### Hemagglutinin titer determination.

Serial 1:2 dilutions of antigen were added to an equal volume of 0.5% chicken red blood cell solution. Following a 1 hour incubation at room temperature plates were read for hemagglutination. HA titer was determined to be the reciprocal of the last well that has hemagglutination.

### Hemagglutinin Inhibition Assay.

Sera were pre-treated by incubation overnight at 37°C with RDEII. After deactivation serial dilutions were mixed with 4HA units of antigen, incubated at RT for 15 minutes, followed by the addition of an equal volume of 0.5% chicken red blood cells. Samples were performed in duplicate and a no antigen control was included to rule out non-specific activity. Plates were read at 1 hour. HAI titer was determined to be the reciprocal of the last well that inhibited hemagglutination.

### RT-PCR of FluA matrix 1 RNA

The lung of each mouse was perfused, removed, homogenized, and total RNA was purified and treated with RNase-free DNase to remove contaminating genomic DNA. Total RNA was reverse transcribed into cDNA using random hexamer primers, and real-time PCR was performed using an ABI 4700 instrument and oligonucleotide primers for M1 RNA, IFN-γ mRNA, and as a positive control, 18S rRNA. M1 RNA and IFN-γ mRNA levels were normalized to 18S rRNA, although the cycle threshold value for 18S rRNA varied less than 10% among the samples.

### Splenocyte restimulation assays.

Splenocytes isolated from vaccinated and control mice were restimulated with antigen. Supernatants were collected after 24-72hrs of stimulation and assayed by ELISA for mouse IFN-γ. Controls included spleen cells cultured 24-72hrs without the addition of specific antigens and unvaccinated splenocytes incubated with the appropriate influenza virus.

### Influenza Challenge of mice

Mice were challenged via intranasal administration of 240 HA units of HKx31 two weeks after the final administration of vaccine. At 4 days following challenge, the mice were sacrificed and tissues collected for histology and RT-PCR to determine extent of protection and cross-protection elicited by the vaccinations.

## RESULTS

**Comparison of influenza vaccination with conventional adjuvants.** CLDC was administered with heat-inactivated HKx31 and compared with alum adjuvanted HKx31 or HKx31 alone. Mice were administered 5.0µg of antigen at Day 0 and 14 and blood samples were evaluated for the humoral immune response at Day 7, 14, 21, and 28 (Figure 2-4). Addition of the CLDC to HKx31 resulted in 10 fold higher levels of IgG (Figure 2), slightly elevated levels of IgG1 (Figure 3), and approximately 100 fold higher IgG2a (Figure 4). Evaluation of HAI titers at day 14 and 28 in subsequent experiments demonstrated an increase in HAI titer as well (Figure 5; p=0.17 with 5 mice per group).

**Dose sparing using inactivated virus.** Cohorts of mice were vaccinated SC on day 0 and 14 with 20µg of CLDC combined with decreasing amounts of heat inactivated HKx31. As can be seen in Figure 6, evaluation of HAI on Day 28 demonstrated that 0.1µg HKx31 with CLDC yielded a greater HAI value than HKx31 alone at 5µg.

**Reduction of influenza A viral load and lung pathology following immunization with CLDC/inactivated virus and subsequent viral challenge.** Cohorts of BALB/c mice were injected IP with 5µg of heat-inactivated virus [either A HKx31 (H3N2) or PR/8/34 (H1N1)] with CLDC either once or twice 2 weeks apart. Mice were challenged with HKx31 two weeks after the final administration of vaccine. At 4 days following challenge, the mice were sacrificed and tissues collected for histology and RT-PCR to determine extent of protection and cross-protection elicited by the vaccinations.

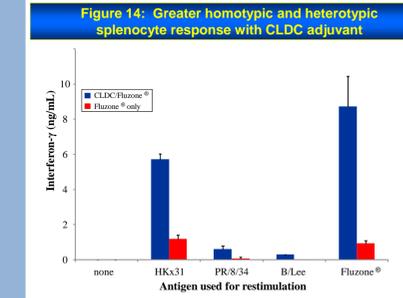
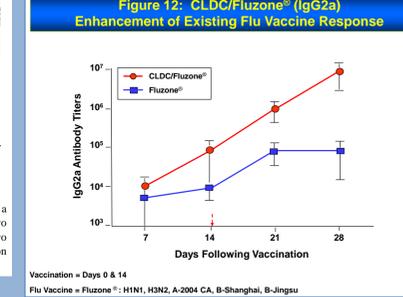
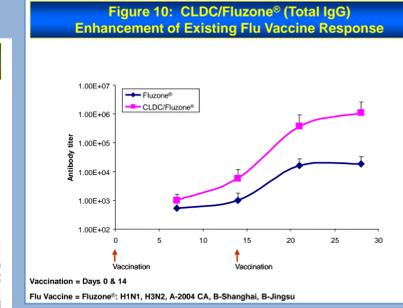
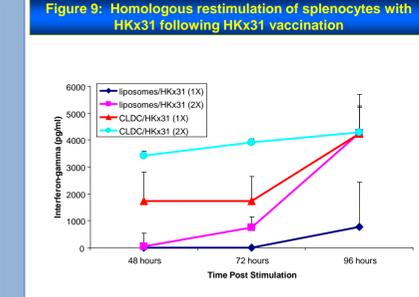
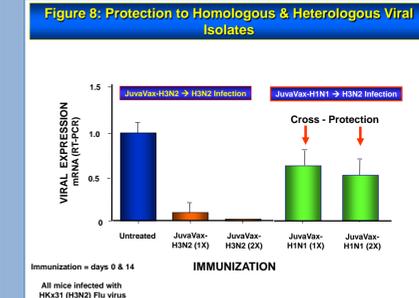
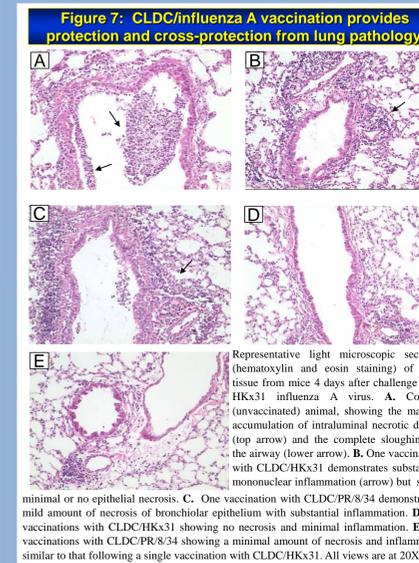
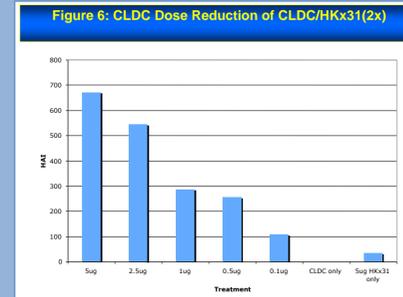
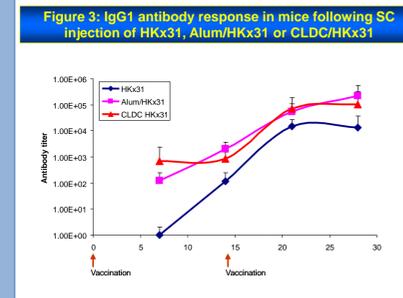
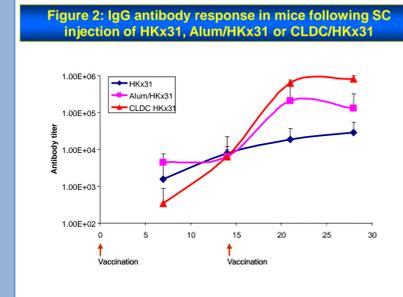
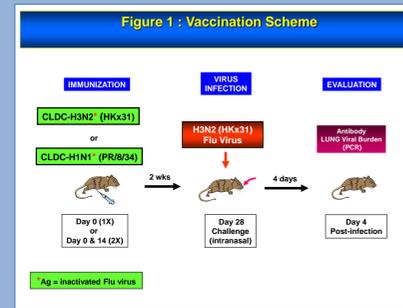
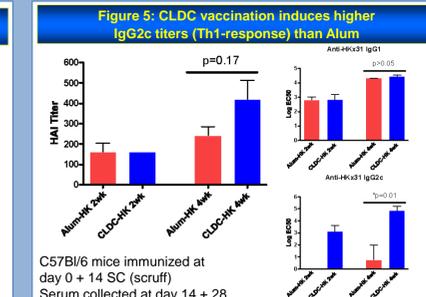
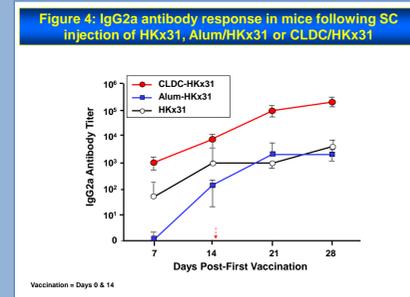
Figure 7A, is a representative of an infected, untreated control mouse. The characteristic feature is the presence of necrosis of epithelial cells lining bronchioles accompanied by perivascular and peribronchiolar inflammation (relative inflammatory score 2-3+). Individual cells lining a bronchiole are necrotic and necrotic debris is accumulated within the airway. Figure 7B shows lung tissue from a mouse that had received a single vaccination with CLDC/HKx31 two weeks prior to challenge. The characteristic feature in these mice was substantial mononuclear inflammatory cell accumulation and scant to no epithelial cell necrosis. The inflammation commonly surrounded blood vessels and closely associated bronchioles (relative inflammatory score 4+). Figure 7C from a mouse that had received a single vaccination with CLDC/PR/8/34 shows only minimal necrosis of bronchiolar epithelium accompanied by substantial inflammation (relative inflammatory score 3). Figure 7D from a mouse vaccinated twice with CLDC/HKx31 shows no necrotic change and minimal mono-nuclear inflammatory cell infiltrate, mainly adjacent to blood vessels and in some focal peribronchiolar areas (relative inflammatory score 1-2+). Figure 7E is from a mouse treated twice with CLDC/PR/8/34 and challenged with HKx31 and shows substantial mononuclear inflammatory cell infiltrate and scant epithelial cell necrosis similar to that seen in the mice vaccinated once with HKx31 and challenged (relative inflammatory score 1+) (20x).

FluA matrix 1 (M1) RNA, as an indicator of viral load was measured in parallel with the lung necropsy material via (RT)-PCR. Figure 8 shows a substantial reduction in viral load following one vaccination with CLDC/HKx31 (P<0.002) and an undetectable level following two vaccinations (P<0.001). Mice vaccinated with PR/8/34 showed a 25% decrease after one vaccination (P = 0.14) and a 40% decrease following a second vaccination (P = 0.05).

**Enhanced humoral immunity by inclusion of CLDC adjuvant with a commercial vaccine.** CLDC complexed with 5.0µg Fluzone® (Sanofi Pasteur) was administered to mice subcutaneously (SC) at day 0, and 14. Fluzone®-specific antibody responses (IgG, IgG1, and IgG2a) were monitored at Day 7, 14, 21, and 28 (Figure 10-12) by ELISA. As expected given the T<sub>H</sub>1 bias of CLDC, the antibody titer of Fluzone®-specific IgG2a was two logs higher than unadjuvanted vaccine (Figure 12). Additionally, IgG1 (Figure 11) and total IgG (Figure 10) were 10-fold and 30-fold higher, respectively, than unadjuvanted vaccine.

**Dose sparing with commercial vaccine.** Cohorts of mice were vaccinated SC on day 0 and 14 with 20µg of CLDC combined with decreasing amounts of Fluzone®. As can be seen in Figure 13, evaluation of HAI on Day 28 demonstrated that 0.1µg Fluzone® with CLDC yielded a greater HAI value than Fluzone® alone at 5µg.

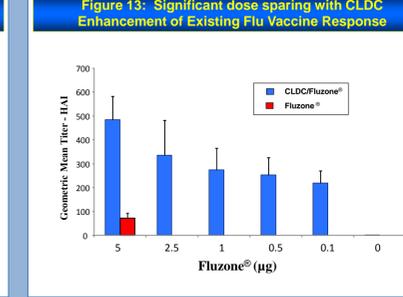
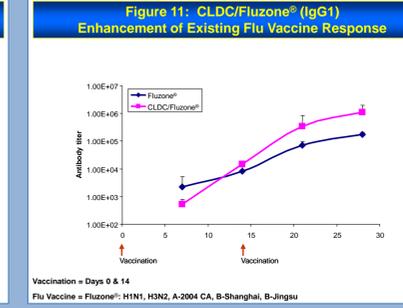
**Increased T-cell response with commercial vaccine.** Splenocytes from the highest dose group in the dose sparing study were isolated and restimulated with Fluzone®, or a representative H1N1 (PR/8/34), H3N2 (HKx31), or B (B/Le/40) influenza. Administration of the vaccine with CLDC resulted in not only a greater recall response when splenocytes were stimulated with vaccine antigen but also when stimulated with unmatched viruses (Figure 14).



**For More Information**

For more information on this poster or the JuvImmune™ adjuvant please contact:

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## CONCLUSIONS

CLDC combined with influenza A antigens provides increased homotypic and heterosubtypic protection as determined by lung histology and viral RNA following challenge. These data are supported by increased antibody titer via ELISA and HAI as well as increased homotypic and heterosubtypic splenocyte responses. Furthermore, the adjuvant has properties that are advantageous for significant dose sparing using existing flu vaccines. The CLDC vaccination strategy results in a substantial induction of both humoral and T-cell mediated immunity, and these results suggest that T-cell mediated immunity alone still provides substantial protection of mice from pulmonary epithelial necrosis and viral replication. Together, these results suggest that CLDC considerably enhances the immune response to existing flu vaccines, most likely by antibody-dependent and antibody-independent mechanisms, directed against surface and internal viral proteins.