Supplementary Methods

1. CaPNP adjuvant dose-response on immunogenicity of CaPNP-adjuvanted inactivated A/CA/04/2009(H1N1pdm) vaccine in mice

This small study describes the dose-response effect of CaPNP adjuvant concentration in an inactivated influenza A (H1N1) 2009 vaccine on immunogenicity (IgG response in particular) in mice. This preliminary study was part of a separate investigation (unpublished) not related to the one described in the manuscript. Some background information and data relevant to the current manuscript is provided below.

Materials

β-propiolactone (BPL)-inactivated, partially purified influenza A/CA/04/2009 (H1N1pdm) virus (HA titer ≥160, Lot #: 58702795; IRR, Cat #: FR-187) was obtained from CDC. HRP conjugated goat anti-mouse IgG, IgG1, and IgG2a were purchased from Sothern Biotech (Birmingham, AL). ELISAMAX Mouse IL-6 ELISA kit was from BioLegend. Peroxidase substrate (OPD) and all reagents and buffer components were either from Fisher Scientific or Sigma.

1.1. Methods

1.1.1. Adjuvant and vaccine formulation

CaPNP-adjuvanted vaccine was formulated using methods modified from previously described [1,2]. Instead of co-precipitating the antigen with the particles during particle formation, particles were manufactured separately and then the inactivated virus was adsorbed to pre-formed CaPNP. Particles were manufactured by mixing inorganic salt solutions of Calcium chloride, sodium citrate, and sodium dibasic phosphate. Salt solutions were combined at ratios to yield nanoparticles with 400-500 nm mean particle sizes [3,4] as determined by Coulter N4Plus Submicron Particle Sizer. CaPNPs produced by this process are stable for at least two years at room temperature without requiring any preservative when stored in tightly capped glass containers. Stability tests during 24 months storage at room temperature or 4^0C indicated no bacterial endotoxin or no significant change in particles size or pH (unpublished data).

Scanning electron microscopy (SEM) imaging of CaPNP indicates substantially spherical morphology (Fig. S1).

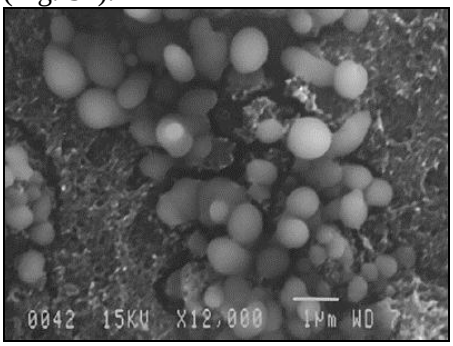

Fig.S1. SEM imaging of CaP nanoparticles.

IIV with or without CaPNP adjuvant were prepared based on a fixed antigen dose of approximately 1.4 µg H1N1 HA. CaPNP-adjuvanted IIV vaccines contained 0.16%, 0.32%, or 0.54% CaPNP (or 0.5 mg/ml Ca^{+2}, 1 mg/ml Ca^{+2}, 1.75 mg/ml Ca^{+2}) in the final formulation. Vaccines were prepared on the day before immunization by mixing the pre-determined amounts of IIV, adjuvant, and sterile water. Vaccine formulations were mixed gently on an end-to-end rotator for 4 hrs at room temperature. Non-adjuvanted IIV and the CaPNP placebo were prepared similarly. Vaccines were stored refrigerated until administered in mice.

1.1.2. Animals and immunization

Pathogen free female BALB/c mice, 18-20 g, were obtained from Charles River Laboratories (Wilmington, MA). Animal husbandry, immunization, and post-immunization procedures were conducted

in accordance with Lampire Biologics (Doylestown, PA) IACUC and in Lampire Small Animal facilities. Groups of five mice were immunized intramuscularly with the following vaccines containing 1.4 µg H1N1 HA or placebo in 100µl volume (50µl in each hind leg): (i) non-adjuvanted IIV as control, (ii) IIV+0.16 % CaPNP, (iii) IIV+0.32% CaPNP, (iv) IIV+0.64% CaPNP, (v) CaPNP placebo.

1.1.3. Antibody responses

Twenty eight days after immunization, blood was collected by retro orbital bleeding under anesthesia. Serum samples from each group were pooled separately. Influenza A (H1N1) 2009-specific IgG was determined by ELISA. As the coating antigen to measure virus specific antibodies, inactivated H1N1 virus was diluted in PBS and 96-well Immulon 2HB microtiter plates were coated with 3µg virus/well based on total protein content and incubated overnight at room temperature. Starting from 1:250 dilutions of pre-bled sera or pooled immune sera from each group and were applied to the plates, serially diluted two-fold in PBS/Tween (PBS containing 0.05% Tween 20), and then incubated for 1.5 h at 37 ◦C. Subsequently, plates were washed and incubated with horseradish peroxidase-conjugated goat antibodies directed against mouse IgG (1:4000) and incubated for 1 h at 37^0C. Plates were washed three times with PBS/Tween and IgGs were detected using o-phenyl diamine (OPD). Plates were developed in the dark for 30 min at room temperature, and then the reaction was stopped by addition of IN H_2SO_4 per well. Absorbance was read at 450nm (A_{450}) using an ELISA reader. Log 2 geometric means of the reciprocal of sera dilutions vs A_{450} values for each vaccine group were plotted (not shown). Log_2 of a serum dilutions were calculated from the following equation:

$$Log_2 (Serum\ Dilution) = [(Log_{10} Serum\ dilution) /(Log_{10} 2)]$$

End-point titers were defined as the highest serum dilution that indicates absorbance value two-fold greater than that of control (non-immune) serum.

2. Results and Conclusion

As shown in Table S1, ~0.3% CaPNP produced the best antibody responses to IIV vaccine in normal mice compared to IIV containing > 0.3% or <0.3% CaPNP. The total IgG produced by IIV+0.32% CaPNP was approximately 3.7-fold higher in magnitude than the IgG produced with the non-adjuvanted IIV. Thus, although not optimized for the study presented in the manuscript, we used 0.3% CaPNP as the best approximation to produce the data for the manuscript.

Table S1. Influenza A (H1N1) 2009 H1N1-specific antibody responses to a single i.m. dose of 1.4µg IIV alone or combined with escalating doses of CaPNP adjuvant

Vaccine	IgG Titer	$IgG_{IIV+CaPNP}/IgG_{IIV}$ Ratio
IIV	12,405	1
IIV+ CaPNP		2.64
0.16% CaPNP	32,734	
0.32% CaPNP	46,291	3.73
0.54% CaPNP	41,720	3.36
CaPNP Placebo	-	-

References

[1] He Q, Mitchell A, Johnson SL, Wagner-Bartak C, Morcol T, Bell SJD. Calcium phosphate nanoparticle adjuvant. Clin. Diagn. Lab. Immunol. 2000;6:899-903.

[2] He Q, Mitchell A, Morcol T, Bell SJD. Calcium Phosphate Nanoparticles Induce Mucosal Immunity and Protection against Herpes Simplex Virus Type 2. Clin. Diagnos Lab. Immunol. 2002;9:1021–1024.

[3] Bell SJD, Morcol T, He Q, Therapeutic calcium phosphate particles and methods of manufacture and use, US Patent 6,355,271 (2002).

[4] Bell SJD, Morcol T, He Q. Therapeutic calcium phosphate particles and methods of manufacture and use. US Patent 8,431,221.