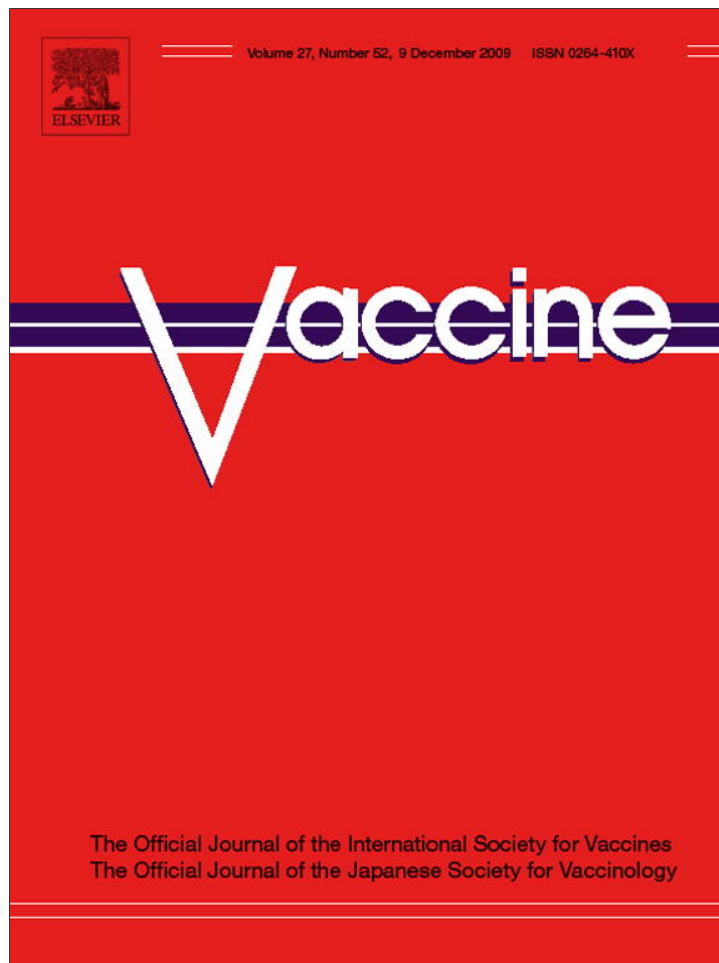


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## Kinetics of viral shedding and immune responses in adults following administration of cold-adapted influenza vaccine

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### ABSTRACT

The optimal type and timing of specimens to study the immune responses to cold-adapted influenza vaccine (CAIV) and shedding of vaccine virus are not well established. Healthy adults were vaccinated with CAIV ( $n=10$ ) or trivalent influenza vaccine (TIV) ( $n=5$ ). Shedding of vaccine strain influenza B was detected by culture in 6 of 10 CAIV recipients; influenza A was also detected in one of these subjects. Viral shedding by quantitative RT-PCR was detected in 9 of 10 subjects. We detected a  $\geq 2$ -fold increase in influenza-specific IgA in nasal wash in 80–100% of CAIV recipients following vaccination, but specific IgG increased in neither nasal wash nor saliva. Recipients of TIV had significant increases in specific serum IgG antibodies. Recipients of both CAIV and TIV had significant increases in IFN $\gamma$ -secreting peripheral blood mononuclear cells (PBMCs). PBMCs from subjects receiving CAIV showed a higher proportion of functional, tissue-tropic T-cells (CD4+CD69+CD18+MIP1 $\alpha$ +) specific for homotypic and heterosubtypic strains of influenza by flow cytometry.

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### 1. Introduction

Vaccination is the most effective means to prevent seasonal influenza infection on a population level [1]. Two formulations of influenza vaccine are licensed for use in the United States: the trivalent inactivated vaccine (TIV), which is administered as an intramuscular injection, and the cold-adapted, live-attenuated influenza vaccine (CAIV), which is administered as an intranasal spray. Because influenza is continuously undergoing antigenic change to escape the host's adaptive immune responses, these vaccines are reformulated every year. Protection induced by TIV correlates with the development of serum antibodies against the surface hemagglutinin of the influenza vaccine strains, whereas protection induced by CAIV is often observed in the absence of a serum antibody response [2], suggesting that other immune pathways, such as mucosal immunity or T-cell mediated immunity contribute to the efficacy of CAIV. CAIV is known to induce significantly greater levels of mucosal IgA antibody, compared with TIV, although protection has also been observed in persons lacking

either a significant mucosal or serum antibody response to CAIV vaccination [3,4].

Few human studies have characterized the cellular immune responses following vaccination with CAIV. The importance of T-cell mediated immunity in clearing influenza virus infection is well established [5–8]. T-cell dependent production of IFN $\gamma$  correlates with viral clearance in murine and human models, and other Th1 cytokines and chemokines, including MIP1 $\alpha$ , are implicated in viral clearance [9]. Priming of T-cells following antigen exposure can induce T-cell expression of tissue-homing receptors, resulting in subsets of T-cells that have tropism for extralymphoid tissues [10]. Vaccination with CAIV may generate specific lymphocytes targeted to the respiratory mucosa, which may contribute to the protection afforded by CAIV in the absence of robust serum antibody responses. In addition, cell-mediated immune responses to cross-reactive epitopes may provide protection against serologically distinct strains of influenza. Studies in children demonstrated that CAIV provides better cross-protection against heterosubtypic strains of influenza than TIV [11–17]. Multiple animal model studies demonstrated a role for T-cell immunity in both long-term protection and heterosubtypic protection against influenza infection. One study in murine influenza found that priming with CAIV resulted in long-lasting heterotypic protection that was T-cell dependent [6]. Circulating lymphocytes that display tissue tropism

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and cross-reactivity may be particularly important in the immune response to influenza infection.

We characterized viral shedding, mucosal and serum antibody responses, and peripheral blood T-cell responses in adults to homotypic and heterosubtypic influenza strains in the early post-vaccination period after CAIV administration.

## 2. Methods and materials

### 2.1. Study design and subjects

We conducted an open-label, 2-arm study in October and early November 2006, in Denver, Colorado. The study protocol was approved by the Colorado Multiple Institutions IRB. All participants provided signed informed consent. Healthy adults aged 18–45 years received the 2006–2007 formulation of CAIV ( $n=10$ ) or TIV ( $n=5$ ). TIV (FluZone; Aventis-Pasteur) was purchased from the pharmacy at the University of Colorado Health Sciences Center and CAIV (FluMist; MedImmune) was provided by MedImmune. Each vaccine contained the recommended strains for the 2006–2007 season: A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004 [18].

Exclusion criteria included allergy to eggs, receipt of influenza vaccine in the 2 years prior to enrollment, immunodeficiency or immunosuppression, serious underlying disease, aspirin therapy, or history of asthma.

### 2.2. Specimen collection/processing

Blood was collected on days 0, 3, 6, 10, 14, and 28 after vaccination. Serum was separated from whole blood and stored at  $-80^{\circ}\text{C}$ . Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Accuspin centrifugation, and cryopreserved in FBS with 10% DMSO. PBMCs were frozen using a rate-controlled freezing container (Nalgene) and subsequently transferred to a  $-140^{\circ}\text{C}$  freezer. Nasopharyngeal (NP) flocked swabs, nasal washes (CAIV recipients only) and saliva specimens were obtained on days 0, 1, 2, 3, 4, 6, and 10 after vaccination. NP swabs and NP washes were obtained via swab of one nare and wash of the other; alternating the type of specimen from nares on successive collection days. Nasal washes were performed by instilling 5 mL of sterile saline into a nostril. One milliliter of recovered nasal wash was placed into 3 mL of M4 medium for culture and the remainder stored at  $-80^{\circ}\text{C}$ . Unstimulated saliva was collected with OraSure kits according to the manufacturer's instructions; after centrifugation of saliva-containing pledgets, specimens were stored at  $-80^{\circ}\text{C}$ . On day 6 after vaccination, 5 of the 10 CAIV recipients received 25 mg of methacholine delivered by intranasal spray 10 min prior to collection of the mucosal specimens.

### 2.3. Viral shedding detected by culture and RT-PCR on nasal swabs and washes

Viral isolation was performed by inoculating 0.3 mL of the clinical specimen each into two tubes of Rhesus Monkey Kidney cells, each from a different vendor (Diagnostic Hybrids and Viromed). Tubes were incubated for up to 14 days. Tubes were examined daily during the first week and thrice weekly thereafter by light microscopy. Hemadsorption of guinea pig red blood cells was performed weekly. All tissue cultures were scraped and stained with specific monoclonal antibodies at the end of the observation period. A positive result was defined as the presence of bright green fluorescence in the cytoplasm of  $\geq 2$  cells. For RT-PCR, RNA was extracted from clinical specimens using a Qiagen kit (Qiagen) and MagnaPure instrument (Roche). Quantitative RT-PCR was performed using a protocol and primers provided by the CDC for influenza

A H1N1, A H3N2 and B. Positive and negative controls, consisting of viral isolates and water, respectively, were included in each run.

### 2.4. Influenza-specific antibody

Influenza strain-specific IgG and IgA were assessed by kinetic ELISA. Plates (Nunc) were coated with attenuated monovalent influenza virus (A/New Caledonia, A/Wisconsin, and B/Malaysia) using a volume corresponding to 1 million TCID<sub>50</sub> per well. After washing and blocking, serial dilutions of sera, nasal wash, or saliva were added. Plates were washed, incubated with horseradish-peroxidase-conjugated affinity-purified goat anti-human IgG or IgA (Jackson ImmunoResearch). Color was developed with ABTS (Sigma) mixed with H<sub>2</sub>O<sub>2</sub> and read at  $\lambda=405$  nm (VersaMax). To correct for variations in sample collection and fluctuations in total antibody levels, nasal and salivary influenza-specific IgG and IgA were expressed as a ratio of specific response to the total amount of IgG or IgA in the same sample. Responses in serum were expressed as a ratio of specific antibody (ELISA units) per total IgG or IgA in the baseline serum sample, as determined by nephelometry. Total IgG and IgA in nasal wash and saliva were determined by kinetic ELISA as above, substituting goat anti-human IgG or IgA (Jackson ImmunoResearch) as the solid phase capture. Standards and controls were included on each plate. Results were accepted if the coefficient of variation for controls was  $<10\%$ .

### 2.5. Influenza strain-specific hemagglutination inhibition (HAI) antibodies

Serum samples from the day of vaccination and 28 days after vaccination were pretreated with receptor-destroying enzyme and then heated to  $56^{\circ}\text{C}$  for 30 min. Serially diluted serum samples in PBS were mixed 1:1 with aliquots of virus (B/Malaysia) corresponding to four HA units, in round-bottom 96-well plates (Corning), and incubated for 15 min at room temperature. Samples were incubated with guinea pig red blood cells for 60 min before reading results. HAI assays were performed at a starting dilution of 1:10, with subsequent serial 2-fold dilutions. The serum HAI antibody titer of a sample was defined as the reciprocal of the last serum dilution with no HA activity. A titer of 2 was assigned to all samples in which the first dilution was negative.

### 2.6. IFN $\gamma$ ELISPOT

IFN $\gamma$  response was assessed by ELISPOT on thawed PBMCs following a 16–20 h in vitro stimulation with attenuated monovalent influenza virus corresponding to the vaccine strains (A/New Caledonia, A/Wisconsin, and B/Malaysia). The concentration of virus stock used for the stimulation was 5 TCID<sub>50</sub>/cell for A/H1N1 and A/H3N2, and 10 TCID<sub>50</sub>/cell for B/Malaysia. Spots were visualized using a CTL ELISPOT plate reader. Background (nonspecific) spots detected in the wells coated with media were subtracted from the quantity of influenza strain-specific spots. Results are reported as spot-forming cells (SFC) per 100,000 PBMCs.

### 2.7. Flow cytometry

T-cell phenotype was assessed by flow cytometry using thawed PBMCs.  $1.0 \times 10^6$  cells were resuspended in RPMI 1640 supplemented with antibiotics and 10% fetal calf serum. Monovalent attenuated influenza virus (B/Malaysia at 10 TCID<sub>50</sub>/cell or the heterosubtypic strain, A/Sydney [H1N1], at 4000 TCID<sub>50</sub>/cell) was added to the cells and specimens were incubated at  $37^{\circ}\text{C}$  with 5% CO<sub>2</sub> overnight in the presence of anti-CD49d and anti-CD5

co-stimulants (3 µg/mL of each). The same volume of media was used for the negative controls. BrefeldinA (Sigma–Aldrich) was added to a final concentration of 10 µg/mL for the last 12–15 h of the incubation. Cells were stained using monoclonal antibodies directed against surface and intracellular molecules: anti-CD3 APC Cy7 (BD BioSciences), anti-CD4 PE TexasRed (Caltag), anti-CD8 PE Cy5.5 (Caltag), anti-CD8 PE Cy7 (Caltag), anti-CD18 FITC (Caltag), anti-CD18 APC (BD BioSciences), anti-CD69 APC (Caltag), anti-CD 69 PE Cy5.5 (Caltag), anti-MIP1α PE (BD Biosciences), and anti-MIP1α APC (BD Biosciences). Cells were treated with Cytofix/Cytoperm solution (BD Biosciences) prior to intracellular staining. Following surface and intracellular staining, cells were washed and fixed with 2% paraformaldehyde in PBS. Specifically stained cells were counted on a MoFlo analyzer (Beckman Coulter). Background (nonspecific) staining detected in cells stimulated with medium only was subtracted from the percentage of influenza strain-specific cells. Results are reported as a percentage of CD3+CD4+ or CD3+CD8+ lymphocytes. The panel of markers was selected to identify activated (CD69+) and functional (MIP1α+) lymphocytes that may also preferentially home to tissues such as lung (CD18+).

For these assays, strain-specific responses were ascertained using monovalent attenuated influenza virus provided by MedImmune (A/New Caledonia [H1N1], A/Wisconsin [H3N2], B/Malaysia, and A/Sydney [H1N1]). The concentrations of virus used for the assays were determined through optimization experiments.

### 2.8. Data analysis

All analyses used non-parametric statistics or log-transformed data. Differences in immune markers between CAIV and TIV recipients were compared using the Dunn's test of multiple comparisons. The Wilcoxon matched pairs test was used for comparison of immune markers at baseline and peak. For multiple comparisons using ANOVA, data were log transformed prior to analysis. A mixed model analysis accounting for repeated measurements was used to identify significant effects on antibody titers measured in different body fluids over multiple days in CAIV and TIV recipients. Analyses were performed using SAS, Microsoft Excel and GraphPad Prism4 software.

## 3. Results

### 3.1. Viral shedding detected by culture and RT-PCR (CAIV recipients only)

By culture, influenza B was isolated in 6 of 10 subjects, one of whom also shed influenza A (Table 1). The median number of days culture-positive was 1 (min 0, max 4). The median day of shedding by culture was day 3 (min 2, max 6).

By quantitative RT-PCR, viral shedding was detected in 9 of 10 subjects (Table 1). Influenza B/Malaysia was detected in 8 subjects and A/New Caledonia in 4 subjects; there was no PCR detection of A/Wisconsin virus. The median number of days PCR-positive by nasal swab was 1 (min 0, max 3) and by nasal wash was 1 (min 0, max 5), and for either was 2.5 (min 0, max 5). The median day of shedding by PCR was day 3 (min 1, max 10). PCR of nasal wash on days 2 and 3 detected 67% of all persons who shed virus, as detected by any combination of the sampling and assay techniques.

Only 1 of 60 PCR assays was positive after day 6. For A/New Caledonia and B/Malaysia, quantitative shedding detected by PCR of nasal swab and nasal wash was significantly higher in culture-positive persons ( $p < 0.0001$ , multiple-comparisons ANOVA; Fig. 1). The mean PCR copy number also correlated with the duration of shedding by culture (Spearman  $r = 0.81$ ,  $p < 0.0001$ ).

**Table 1** Influenza culture results and RNA PCR copy number (copies/mL) from nasal swab and nasal wash from 1 to 10 days following CAIV. All PCR results shown are for B/Malaysia, except in those highlighted in gray which represent A/New Caledonia (■=A/New Caledonia). Boxes with two values indicate the presence of two different virus strains in the sample. K = 1000.

Patient	Day 1			Day 2			Day 3			Day 4			Day 6			Day 10			
	Culture	Swab PCR	Wash PCR	Culture	Swab PCR	Wash PCR	Culture	Swab PCR	Wash PCR	Culture	Swab PCR	Wash PCR	Culture	Swab PCR	Wash PCR	Culture	Swab PCR	Wash PCR	
1		14K																	
2																			
3								A Swab	0.4K	2K	B Wash	11K							
4				B Swab	49K	28K			85K	0.7K		5K							
5																			
6		0.2K			4K														
7					2K														0.9K
8						9K				10K						B Wash		7K	
9						0.5K													
10					12K	16K		B Swab	5K	15K		3K							
						19K		B Wash	21K	45K		34K				B Both	19K		9K

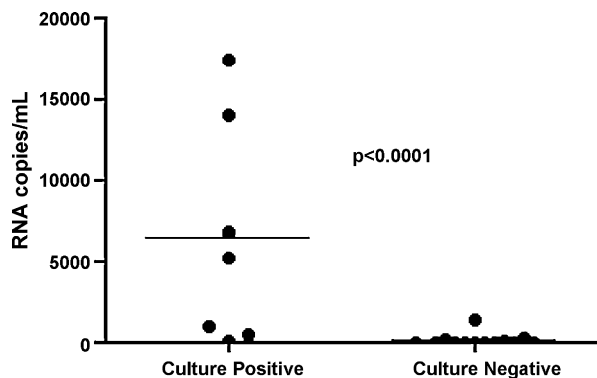


Fig. 1. Average nasal influenza PCR copy-number in culture-positive vs culture-negative adults, within 10 days following receipt of CAIV.

Using any positive result (culture or PCR) as the gold standard, the sensitivity of nasal swab and nasal wash PCR was 70% and 90%, respectively. The viral copy number detected by PCR of nasal wash or nasal swab specimens did not significantly differ ( $p=0.44$  for B/Malaysia and  $p=0.68$  for A/New Caledonia, by multiple-comparisons ANOVA).

3.2. Antibody responses in serum, nasal wash, and saliva following influenza vaccination

The peak levels of serum IgA specific for influenza A/New Caledonia and B/Malaysia, but not A/Wisconsin, increased after CAIV. In contrast, strain-specific serum IgG did not significantly increase after CAIV vaccination. The majority of TIV recipients had a  $\geq 2$ -fold increase in strain-specific serum IgG. (Tables 2 and 3).

Serum antibodies to influenza B/Malaysia were measured by both ELISA and HAI for all participants. Twenty-eight days after vaccination, significant increases of HAI-measured antibodies (defined as a  $\geq 4$ -fold increase in HAI titer) were observed in 1 (10%) of 10 CAIV recipients and 3 (60%) of 5 TIV recipients. There was a significant correlation between HAI- and ELISA-measured antibody fold-rises (Spearman  $r$ -value of 0.54,  $p=0.04$ ).

In nasal wash (measured in CAIV recipients only), strain-specific IgA increased by  $\geq 2$ -fold from baseline in 100% of subjects for A/New Caledonia, 80% for A/Wisconsin, and 100% for B/Malaysia (Table 2). A lower proportion experienced a 2-fold rise in strain-

specific IgG. The day of peak mucosal antibodies ranged from day 1 to day 10 (median day 6 for IgG and day 4 for IgA). Peak secretion did not coincide for all viruses in the same individuals. Comparison of baseline and peak antibody levels after CAIV administration demonstrated an increase in strain-specific IgA for all three viruses and IgG for A/New Caledonia and A/Wisconsin (Table 3).

In saliva there were no significant differences between baseline and peak anti-influenza IgG or IgA in CAIV or TIV recipients. Three (60%) TIV recipients had a  $\geq 2$ -fold increase in saliva IgG titers against each of the influenza strains in the vaccine. The administration of intranasal methacholine did not increase the yield of total or specific IgG or IgA in nasal wash or saliva ( $p > 0.1$  for all).

Among TIV recipients, influenza-specific IgG levels in serum showed strong associations with those in saliva, with a median coefficient of correlation of 0.83 and a significant effect of serum antibody levels on saliva antibody levels in the mixed model analysis ( $p < 0.001$ ). In contrast, serum influenza-specific IgA levels had no significant association with or effect on nasal wash influenza-specific IgA levels in CAIV recipients.

Because eight CAIV recipients shed influenza B/Malaysia, but none shed A/Wisconsin, we compared baseline serum and nasal wash antibody levels against these two strains in the CAIV recipients. The baseline nasal wash B/Malaysia-specific IgA was low (0.47 [95% CI: 0.14, 0.79]) compared to A/Wisconsin-specific IgA (1.68 [95% CI: 1.7, 2.9]),  $p=0.02$  (Table 3). The baseline serum influenza B-specific IgG was 11.2 (95% CI: 5.3, 17.0) vs. 8.2 (95% CI: 3.3, 13.0) for A/Wisconsin ( $p=0.38$ ). There was no correlation between the duration of shedding (by culture and/or PCR) and the baseline or peak levels of nasal wash virus-specific IgG or IgA for B/Malaysia or A/New Caledonia.

3.3. T-cell responses to influenza vaccination

Influenza strain-specific IFN $\gamma$ -secreting PBMCs trended upward following both CAIV and TIV for all three viruses (Fig. 2). Peak IFN $\gamma$ -response occurred at days 10–14. Among CAIV recipients, statistically significant increases were seen between pre-vaccination and peak post-vaccination IFN $\gamma$  responses ( $p=0.002$  for all three viruses, by Wilcoxon ranked sums test). Among TIV recipients, the increases between pre-vaccination and peak post-vaccination IFN $\gamma$  cells did not reach statistical significance due to the smaller sample size. The magnitude of the IFN $\gamma$  response did not differ between

Table 2  
CAIV and TIV recipients with  $\geq 2$ -fold increase in the influenza strain-specific antibody in nasal wash, saliva, and serum, as measured by kinetic ELISA. ND = not done.

	Nasal wash IgG, n (%)			Nasal wash IgA, n (%)			Saliva IgG, n (%)			Saliva IgA, n (%)			Serum IgG, n (%)			Serum IgA, n (%)		
	H1N1	H3N2	B	H1N1	H3N2	B	H1N1	H3N2	B	H1N1	H3N2	B	H1N1	H3N2	B	H1N1	H3N2	B
CAIV	4 (40)	3 (30)	2 (20)	10 (100)	8 (80)	10 (100)	4 (40)	2 (20)	3 (30)	0 (0)	2 (20)	2 (20)	2 (20)	0 (0)	1 (10)	1 (10)	0 (0)	3 (30)
TIV	ND	ND	ND	ND	ND	ND	3 (60)	3 (60)	3 (60)	1 (20)	0 (0)	0 (0)	4 (80)	3 (60)	4 (80)	2 (40)	2 (40)	3 (60)

Table 3  
Influenza-specific antibody in nasal wash, saliva, and serum at baseline and peak following vaccination. Results are reported as the ratio of specific antibody to total IgG or IgA, measured in ELISA units.  $p$ -Value given for difference between baseline and peak, by Wilcoxon signed-rank test. ND = not done. Statistically significant changes are in bold text.

	Nasal wash IgG			Nasal wash IgA			Saliva IgG			Saliva IgA			Serum IgG			Serum IgA		
	H1N1	H3N2	B	H1N1	H3N2	B	H1N1	H3N2	B	H1N1	H3N2	B	H1N1	H3N2	B	H1N1	H3N2	B
CAIV (n = 10)																		
Baseline	0.16	0.31	0.61	1.03	1.68	0.47	.70	0.33	0.89	0.37	0.62	0.18	3.46	8.17	11.15	1.22	1.34	0.73
Peak	<b>0.24</b>	<b>0.48</b>	<b>0.57</b>	<b>5.03</b>	<b>11.67</b>	<b>2.48</b>	<b>1.07</b>	<b>0.56</b>	<b>1.25</b>	<b>0.30</b>	<b>0.84</b>	<b>0.38</b>	<b>5.22</b>	<b>8.11</b>	<b>13.32</b>	<b>1.51</b>	<b>1.30</b>	<b>1.10</b>
$p$ -Value	<b>0.05</b>	<b>0.01</b>	<b>1</b>	<b>0.002</b>	<b>0.006</b>	<b>0.002</b>	0.32	0.06	0.49	0.1	0.23	0.74	0.77	0.77	<b>1</b>	<b>0.02</b>	0.85	<b>0.02</b>
TIV (n = 5)																		
Baseline	ND	ND	ND	ND	ND	ND	0.56	0.32	0.59	0.37	0.96	0.38	2.99	2.83	3.43	0.93	1.87	1.24
Peak	ND	ND	ND	ND	ND	ND	0.98	0.80	0.89	0.61	0.94	0.51	7.73	6.80	8.61	2.12	4.27	2.42
$p$ -Value							0.43	0.06	0.43	0.18	0.44	0.62	0.06	0.06	0.06	0.13	0.31	0.06

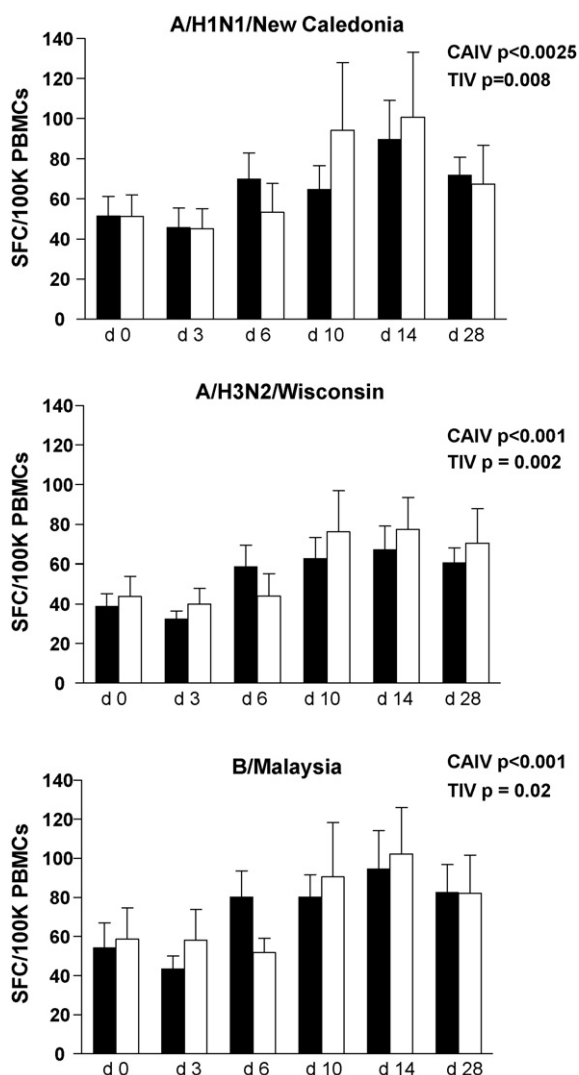


Fig. 2. PBMC influenza strain-specific IFN $\gamma$ -ELISPOT at baseline (d 0) and following vaccination with CAIV (■) or TIV (□). *p*-Value for trend is shown. d = day.

CAIV and TIV recipients at any time point. Among CAIV recipients, the maximum fold rise in IFN $\gamma$  response was highest in individuals with the lowest baseline level. This inverse correlation was statistically significant for A/New Caledonia ( $r = -0.68$ ,  $p = 0.03$ ) and B/Malaysia ( $r = -0.84$ ,  $p = 0.003$ ).

The highest morbidity of influenza disease derives from pulmonary complications. Based on previous work [19–21], we used the expression of CD18 in association with MIP1 $\alpha$  and CD69 to identify T-cells that were activated and produced Th1 chemokines in response to in vitro influenza stimulation, which may have preferential migration or homing to the lung.

At day 14, the proportion of CD4+CD69+CD18+MIP1 $\alpha$  cells specific for B/Malaysia was 0.10% vs. 0.09% in CAIV and TIV recipients, respectively ( $p = 0.95$ ), and the proportion of cells specific for A/Sydney was 1.4% vs. 1.1% in CAIV and TIV recipients, respectively ( $p = 0.62$ ). The proportion of CD8+CD69+CD18+MIP1 $\alpha$  cells specific for B/Malaysia was 0.25% vs. 0.46% in CAIV and TIV recipients, respectively ( $p = 0.71$ ), and the proportion of cells specific for A/Sydney was 2.88% vs. 2.36% in CAIV and TIV recipients, respectively ( $p = 0.62$ ).

At day 28, the proportion of CD4+CD69+CD18+MIP1 $\alpha$  cells specific for B/Malaysia was 0.22% vs. 0.01% in CAIV and TIV recipients, respectively ( $p = 0.02$ ), and the proportion of cells specific for

A/Sydney was 0.98% vs. 0.13% in CAIV and TIV recipients, respectively ( $p = 0.03$ ). The proportion of CD8+CD69+CD18+MIP1 $\alpha$  cells specific for B/Malaysia was 0.25% vs. 0.004% in CAIV and TIV recipients, respectively ( $p = 0.04$ ), and the proportion of cells specific for A/Sydney was 0.04% vs. 0.001% in CAIV and TIV recipients, respectively ( $p = 0.20$ ) (Fig. 3).

#### 4. Discussion

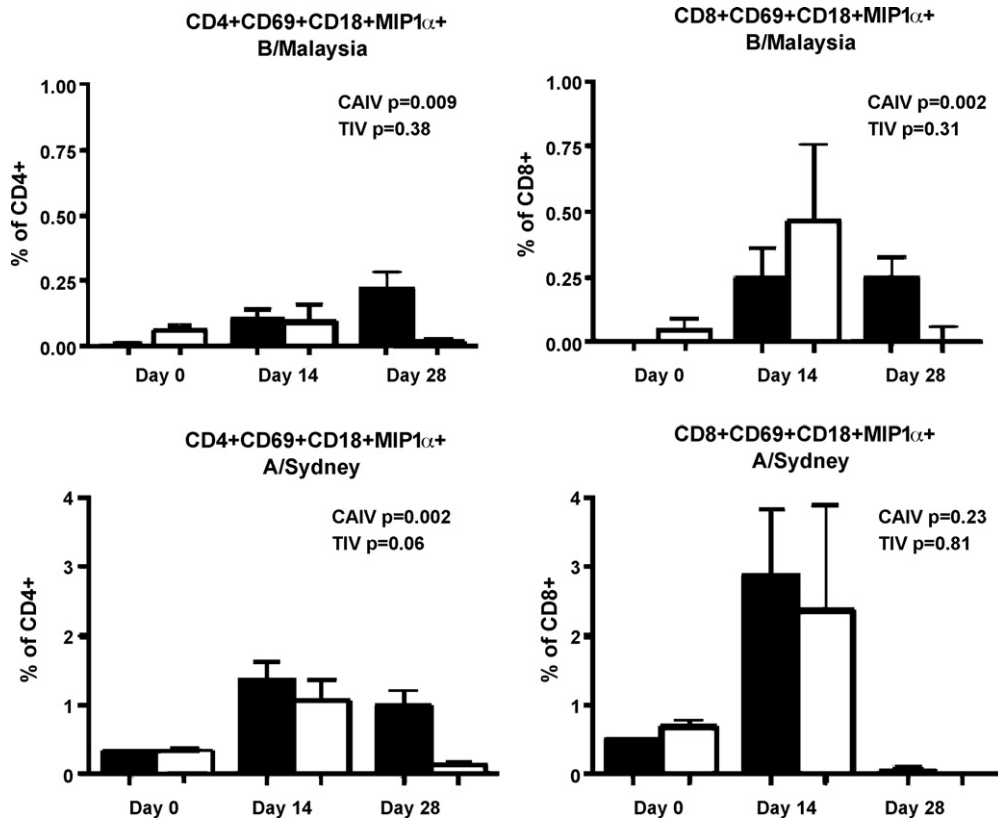
We report on the kinetics and characteristics of responses to vaccination with CAIV, which will inform the design of future immunogenicity studies and provide insight on the mechanism of heterosubtypic protection. Through use of PCR, we detected influenza viral shedding in a high percentage of CAIV recipients. We found that baseline nasal IgA was associated with absence of shedding and that vaccination with CAIV-induced cross-protective T-cells with an activated, functional and tissue tropic phenotype. Data suggest that immunity induced by natural influenza infection may be longer lived and broader than that induced by inactivated vaccine [22,23]. Vaccination with live-attenuated influenza virus by an intranasal route may induce local and cell-mediated immune responses that better mimic the multiple immune mechanisms of protection afforded by natural infection.

Variability in detection of shedding may be influenced by the timing and methods used for sampling, as well as for detection of virus. We detected viral shedding in a higher percentage of CAIV recipients (60% by culture and 90% by PCR) than has been reported by other studies. Among adults given CAIV, Talbot et al. documented culture-proven shedding in 50% of individuals on day 3 following vaccination [24]. King et al. found shedding in 1 (4%) of 28 HIV-infected participants and none of 27 HIV-uninfected participants [25] following CAIV. In another larger study, culture-proven shedding was documented in 17% of healthy adults who received CAIV [26]. Our study incorporated frequent sampling, collection of both nasal swab and nasal wash specimens, and used both culture and PCR to measure shedding. These factors likely account for the differences in our results compared to other published findings. Of note, despite shedding, transmission of vaccine viruses after CAIV has been documented only once [27].

In this small sample, the sensitivity of RT-PCR for influenza using either nasal wash or nasal swab was similar. This confirms reports comparing RT-PCR of nasal swab vs. nasal wash or nasal aspirate to detect influenza in children with respiratory symptoms [28,29]. We found that virus was detected by culture less often than by PCR. The average PCR copy number was significantly higher among persons with positive cultures, although it was not possible to establish a quantitative viral load cut-off that corresponded to culture positivity. For detection of infection, nasal flocced swab specimens appear to offer adequate sensitivity and excellent ease of collection. However, nasal wash specimens allow for measurement of nasal antibodies and cytokines, which may be important for evaluation of vaccine immunogenicity. Based on our results, the peak of shedding would best be detected by PCR analysis of specimens collected on post-vaccination day 3, although a more complete picture of shedding would be captured by collecting specimens on post-vaccination days 2–4.

CAIV recipients exhibited minimal antibody responses in serum, as measured by HAI and the more sensitive ELISA for influenza strain-specific IgG and IgA. Low serum antibody responses to CAIV are consistent with many previous reports in which a  $\geq 4$ -fold increase in HAI was documented in 0–39% of adult recipients of CAIV, depending on the strain and the baseline serostatus [4,25,28,30].

We confirmed that CAIV recipients had low but detectable influenza-specific nasal IgA response, but not a salivary antibody



**Fig. 3.** Frequency of CD4+ (left column) and CD8+ (right column)-T-cells with the CD69+CD18+MIP1 $\alpha$ + phenotype measured by flow cytometry at baseline and following vaccination with CAIV (■) or TIV (□). PBMCs were stimulated in vitro with either homotypic (B/Malaysia) or heterosubtypic (A/Sydney) influenza virus. The frequency of CD69+CD18+MIP1 $\alpha$ + is expressed as a percent of the parent T-cell population (CD4+ or CD8+). *p*-Value given for baseline (day 0) vs. peak response, by Wilcoxon ranked sums test.

response. The mucosal response to a monovalent cold-adapted influenza vaccine was reported by Moldoveanu et al. as an increase in specific nasal antibody detected on day 13 (peak measured on day 28), but without any significant change in specific salivary antibody [31]. Thus, although salivary specimens may be easier to collect, they are not a surrogate for the assessment of the intranasal mucosal antibody production. Furthermore, the strong association between saliva and serum anti-influenza antibodies suggests that salivary antibodies are primarily a transudate. In contrast, the lack of association between nasal wash and serum IgA antibodies suggests that nasal wash IgA antibody levels measure local production.

Inhalation of methacholine increases nasal secretion of certain substances, but there are conflicting reports regarding the effects of methacholine on nasal antibody secretion [32–34]. Because mucosal antibody levels are typically low and can be difficult to measure, we sought to determine if methacholine use would increase antibody yield. We found that administration of inhaled methacholine did not enhance detection of specific antibody levels in nasal wash or salivary specimens.

The significant role of T-cell mediated immunity in the defense against influenza is well established in the mouse model [6,35,36], but its role in humans has been more difficult to demonstrate [37]. Belshe et al. showed that children without influenza-specific antibodies in serum or nasal wash were protected against the disease [5], suggesting that the mechanism of protection was a function of T-cell mediated immunity. Others also showed that vigorous T-cell responses are associated with reduced morbidity of influenza infections [7,38]. We determined that both TIV and CAIV boosted IFN $\gamma$  responses significantly and comparably. The magnitude of the ELISPOT-measured responses to CAIV was negatively associated with the pre-vaccination T-cell response, which confirms previous

reports [39]. He et al. [40], using a flow cytometric assay found no increase in IFN $\gamma$  T-cell responses in adults after CAIV or TIV vaccination. The higher sensitivity of ELISPOT assays compared with the flow cytometric ones [41] may explain this difference.

Unlike antibody responses, anti-influenza T-cell responses are directed predominantly against conserved gene products, providing heterotypic cross-protection [36,42–44]. In this study, we showed both CAIV and TIV recipients developed heterosubtypic-specific T-cell responses; however, these cells were longer lasting in CAIV recipients compared with TIV recipients (Fig. 3). These results support the concept that live-attenuated vaccines generate more vigorous T-cell responses than inactivated vaccines. Several clinical investigations showed that CAIV conferred protection against heterosubtypic strains of wild type influenza [5,12–17]. This effect was more prominent in children and could not always be demonstrated in adults [45].

Another important aspect of the anti-influenza immune responses is the ability to confer protection against lower respiratory tract infection, which is responsible for major morbidity. Homing receptors for the respiratory tract in general, and lungs in particular, have been extensively sought for, but specific receptors, such as the ones for skin and gut, have not yet been identified. Using the CD18 portion of LFA-1, which was previously shown to be expressed in abundance by memory T-cells in the lungs [19,21], we showed that both CAIV and TIV recipients developed influenza-specific Th1 T-cells with this putative pulmonary homing receptor. This response was maintained through 28 days in CAIV recipients. It will be important to determine in future studies whether the heterotypic T-cell responses generated by either vaccine correlate with protection against heterotypic wild type influenza virus.

Due to the high percentage of shedding and our small sample size, we were not able to evaluate many factors associated with protection from shedding. However, we found that baseline nasal wash strain-specific IgA was higher for A/Wisconsin, which was not shed by participants, than for B, which was shed by 8/10 participants. These data are consistent with those from other nasal vaccine studies in which specific nasal IgA was associated with resistance to shedding and/or with protection from illness. These data suggest that stimulation of mucosal antibody production is one mechanism by which CAIV may confer protection against influenza or serve as a correlate of protection [11,24,46,47].

This study was limited by the small number of subjects enrolled and the brief duration of sample collection, particularly for mucosal antibodies. This is primarily a descriptive study of the kinetics of shedding and immune responses to CAIV; comparisons between recipients of CAIV and TIV are purely exploratory. Although we limited participants to healthy persons who had not received influenza vaccine in the prior 2 years, the immune response to vaccination is likely affected by prior infection or vaccination. This analysis did look at changes in pre–post-vaccine antibody levels, but did not control for pre-existing antibody in other ways.

The importance of understanding immunity to influenza is underscored not only by the thousands of deaths that are annually attributable to this virus, but also by the threat of pandemic influenza. Natural infection elicits protection through a variety of immune mechanisms. By mimicking natural infection through mucosal inoculation and viral shedding, CAIV may better stimulate these alternative immune pathways, compared with inactivated vaccine. A better understanding of the correlates of CAIV-induced protection will be critical to the development of more effective vaccines.

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