



## Short communication

## Comparison of humoral and cellular immune responses to inactivated swine influenza virus vaccine in weaned pigs

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## ABSTRACT

Humoral and cellular immune responses to inactivated swine influenza virus (SIV) vaccine were evaluated and compared. Fifty 3-week-old weaned pigs were randomly divided into the non-vaccinated control group and vaccinated group containing 25 pigs each. Pigs were vaccinated intramuscularly twice with adjuvanted UV-inactivated A/SW/MN/02011/08 (MN/08) H1N2 SIV vaccine at 6 and 9 weeks of age. Whole blood samples for multi-parameter flow cytometry (MP-FCM) and serum samples for hemagglutination inhibition (HI) assay were collected at 23 and 28 days after the second vaccination, respectively. A standard HI assay and MP-FCM were performed against UV-inactivated homologous MN/08 and heterologous pandemic A/CA/04/2009 (CA/09) H1N1 viruses. While the HI assay detected humoral responses only to the MN/08 virus, the MP-FCM detected strong cellular responses against the MN/08 virus and significant heterologous responses to the CA/09 virus, especially in the CD4+CD8+ T cell subset. The cellular heterologous responses to UV-inactivated virus by MP-FCM suggested that the assay was sensitive and potentially detected a wider range of antigens than what was detected by the HI assay. Overall, the adjuvanted UV-inactivated A/SW/MN/02011/08 H1N2 SIV vaccine stimulated both humoral and cellular immune responses including the CD4–CD8+ T cell subset.

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## 1. Introduction

Swine influenza virus (SIV) is a negative strand, segmented, enveloped RNA virus of the *Orthomyxoviridae* family. Influenza infection in swine is recognized clinically as an acute respiratory disease characterized by fever, coughing, lethargy, anorexia and nasal discharge (McQueen et al., 1968; Alexander and Brown, 2000; Richt et al., 2003). Over the past ten years, vaccinating against SIV

has become a common practice in the US swine industry. However, the development of efficacious, cross-reactive vaccines has been challenged by the rapid change in SIV subtypes due to reassortment and antigenic drift. Inactivated vaccines, commercially available in the US since 1994, have been shown to play a significant role in preventing SIV infection through the generation of anti-SIV antibodies (Bikour et al., 1996). In addition, hemagglutination inhibition (HI) antibody titers in the serum have been used to predict clinical protection against challenge virus. However, HI titers against homologous virus may provide only partial protection against heterologous challenge (Vincent et al., 2008).

Protective immunity against infection with influenza involves both the humoral and cell-mediated immune

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(CMI) response. Many studies investigating the immune response to influenza virus have been conducted in mice. Collectively, these studies have shown that a combination of neutralizing antibodies, mucosal immune responses and T cells are important for protection and recovery from disease (Tamura et al., 2005). Cell-mediated immune responses that include CD4<sup>+</sup> T helper cells and CD8<sup>+</sup> cytotoxic T cells have also been shown to be important for protection or recovery against heterosubtypic challenge in mice, respectively (Liew et al., 1984; Moran et al., 1999; Nguyen et al., 1999, 2001). In addition, IgA has been shown to be more cross-reactive against heterologous challenge than IgG in mice (Tamura et al., 1991). Although reports describing the immune response to influenza vaccines or infection are limited in the swine host; humoral immune responses that include both IgA and IgG at the mucosal level have been shown to be important for protecting the respiratory tract from SIV in swine (Larsen et al., 2000).

Future control of influenza A viruses in swine will depend on the development and use of vaccines that provide adequate cross-protection and the induction of an immune response based on both humoral and cell-mediated mechanisms. Collectively, influenza studies in a natural host, such as swine, are limited and minimal information regarding the humoral and CMI responses elicited by inactivated vaccines are available. The objectives of this study were to evaluate and compare the humoral and cellular responses to homologous and heterologous viruses following inactivated SIV vaccination, using standard HI assays and multi-parameter flow cytometry (MP-FCM). The MP-FCM has been well established in our laboratory to measure specific CMI responses to viral and bacterial antigens in bovine, porcine, and equine systems using 4–6 color combinations (Charerntantanakul et al., 2006a, 2006b; Platt et al., 2008, 2009, 2010a, 2010b). This study reports our current porcine 6-color MP-FCM.

## 2. Materials and methods

### 2.1. Experimental animals and vaccine

Fifty 3-week-old pigs from a herd free of SIV and PRRSV were used in the study. These pigs were part of the study of Gauger et al. (submitted for publication). Pigs were screened for influenza A nucleoprotein antibody by ELISA (Multi-S ELISA, IDEXX Laboratories, Inc., Westbrook, ME) prior to the study to ensure the absence of SIV specific antibody. To reduce confounding effects of respiratory tract bacterial co-infections, pigs were treated with ceftiofur crystalline free acid (Pfizer Inc., New York, NY) at three weeks and tulathromycin injectable solution (Pfizer Inc., New York, NY) at 8 days before first vaccination according to manufacturer recommendations. The pigs were randomly divided into two groups. Pigs in the control group ( $n=25$ ) were not vaccinated. Pigs in the vaccinated group ( $n=25$ ) were inoculated intramuscularly with 2 ml UV-inactivated A/SW/MN/02011/08 H1N2 SIV vaccine ( $10^{5.7}$  TCID<sub>50</sub>/ml) with an emulsified oil-in-water adjuvant (Emulsigen®-D, MVP Technologies, Omaha, NE) at 6 and 9 weeks of age. The pigs were housed at the National

Animal Disease Center (NADC) and cared for in compliance with the Institutional Animal Care and Use Committee.

### 2.2. Recall antigens

The homologous vaccine strain A/SW/MN/02011/2008 H1N2 (MN/08) (a  $\delta$ -cluster H1 SIV) and the heterologous pandemic A/CA/04/2009 H1N1 (CA/09) (a 2009 pandemic H1 SIV) were propagated in MDCK cells to approximately  $10^{5.7}$  TCID<sub>50</sub>/ml. The viruses in media were inactivated using the sterilize setting in an ultraviolet cross-linking chamber (GS Gene Linker, Bio-Rad, Hercules, CA). Culture media supernatant from uninfected MDCK cells served as mock antigen.

### 2.3. Blood samples

Whole blood samples were collected 23 days after second vaccination in vacutainer cell preparation tubes with sodium citrate (CPT™, cat # 362761, BD Diagnostics, Franklin Lakes, NJ). The peripheral blood mononuclear cells (PBMC) were isolated within 2 h of collection and activated within the same day. Serum samples were separated from blood collected into serum separator tubes on day 28 after second vaccination and utilized in the HI assay.

### 2.4. Hemagglutination inhibition assay

Sera used in the HI assay were heat-inactivated at 56 °C for 30 min. Non-specific HA inhibitors were removed with a 20% suspension of kaolin (Sigma Aldrich, St. Louis, MO, USA) and natural serum agglutinins were removed by adsorption with 0.5% turkey red blood cells (RBCs). The HI assay was performed against the homologous MN/08 and heterologous CA/09 viruses with turkey RBC using standard techniques (Palmer et al., 1975). Reciprocal titers for HI assays were log<sub>2</sub> transformed for statistical analysis and reported as geometric means.

### 2.5. Multi-parameter flow cytometry

#### 2.5.1. Peripheral blood mononuclear cell isolation

The CPT tubes were centrifuged at  $1500 \times g$  for 30 min at room temperature ( $\sim 25^\circ\text{C}$ ) within 2 h after blood collection. The PBMC (2–3 ml) were collected into 15 ml conical centrifuge tubes. The red blood cells were lysed with 6 ml buffered water (deionized water with 0.15 M Na<sub>2</sub>HPO<sub>4</sub> and 0.15 M KH<sub>2</sub>PO<sub>4</sub>) for 90 s followed by 3 ml of  $3 \times$  PBS. The tubes were centrifuged at  $700 \times g$  for 10 min and the PBMC were washed in 10 ml PBS. After centrifugation, the PBMC were resuspended in 3 ml RPMI++ (RPMI 1640 (Mediatech, Inc., Herndon, VA) with 15% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 1.5% penicillin/streptomycin/amphotericin B solution (Mediatech, Inc., Herndon, VA) and 1.5  $\mu\text{g/ml}$  gentamicin (Gibco, Invitrogen Co., Grand Island, NY)). The PBMC suspensions were counted and  $10^7$  cells of each sample were diluted with RPMI++ to 2 ml ( $5 \times 10^6$  cells/ml) in new tubes.

### 2.5.2. PBMC activation

PBMC were activated in 96-well flat-bottomed tissue culture microtiter plates (Falcon, BD Labware, Franklin Lakes, NJ), 7 wells for each sample. One well received 50  $\mu$ l RPMI++ as non-antigen stimulation control. The second well received 50  $\mu$ l ConA at 5  $\mu$ g/ml final concentration as mitogen control. The third well received 50  $\mu$ l undiluted MDCK media and served as the mock-stimulation control. Each strain of undiluted UV-inactivated SIV was added to duplicate wells as SIV-stimulation wells. Two hundred microliters of each PBMC suspension with  $10^6$  cells were added to each of 7 wells and the plates were incubated in a 5% CO<sub>2</sub> humidified incubator at 37 °C.

### 2.5.3. PBMC staining

Four days after activation, in order to block protein transport, brefeldin A (50  $\mu$ l) (BD Biosciences Pharmingen, San Diego, CA) was added to 96-well round-bottomed tissue culture microtiter plates (Falcon, BD Labware, Franklin Lakes, NJ) at the concentration recommended by the manufacturer, in the same pattern as the PBMC activation plates. The PBMC were then transferred correspondingly and mixed well with brefeldin A. The plates were incubated for an additional 4 h.

Plates were then centrifuged at  $430 \times g$  for 1 min and the supernatants removed by a firm rapid flicking of the plates. The PBMC were washed once with 100  $\mu$ l PBS++ (PBS with 0.5% bovine serum albumin, and 0.1% sodium azide). All washing steps and antibody removal from the plates were by the same centrifugation and plate flicking cycle. All antibodies used in the staining were previously titrated for their optimum concentrations in the same PBMC setting (data not shown). The primary antibody mix for surface antigens (50  $\mu$ l) was added to all PBMC wells except the secondary antibody control wells and incubated at room temperature (RT, 25 °C) for 15 min. The mAb mix consisted of mouse anti-swine CD4 (IgG2b, cat# 74-12-4, VMRD, Inc., Pullman, WA), mouse anti-swine CD8 $\alpha$  (IgG2a, cat# 76-2-11, VMRD, Inc., Pullman, WA), rat anti-swine  $\gamma\delta$  TCR (Rat IgG2a, cat# 551543, BD Pharmingen, San Diego, CA), mouse anti-swine CD25 (IgG1, cat# PGBL25A, VMRD, Inc., Pullman, WA) in PBS++. The primary antibody mix was removed after incubation and the cells were washed twice with 100  $\mu$ l PBS++. The secondary antibody mix for surface antigens (50  $\mu$ l) was added to all PBMC wells including the secondary antibody control wells and incubated at room temperature for 15 min. The secondary antibody mix consisted of goat anti-mouse IgG2b-AF647 (Alexa Fluor 647, cat# A21242, Invitrogen, Carlsbad, CA), goat anti-mouse IgG2a-PE (Phycoerythrin, cat# P21139, Invitrogen, Carlsbad, CA), goat anti-rat IgG2a-FITC (cat# STAR113F, AbDSerotec, Raleigh, NC), and goat anti-mouse IgG1-PE-TR (Phycoerythrin-Texas Red, cat# M32017, Invitrogen, Carlsbad, CA) in PBS++. The secondary antibody mix was removed after incubation and the cells were washed three times with 100  $\mu$ l PBS++. For intracellular staining, the PBMC were treated with 100  $\mu$ l BD cytofix-cytoperm solution (BD Biosciences Pharmingen, San Diego, CA) at RT for 30 min and washed once with BD perm-wash solution (BD Pharmingen, San Diego, CA). The intracellular primary antibody mix, consisted of rabbit anti-swine IFN- $\gamma$  (polyclonal

antibody, cat# ASC4032, Invitrogen, Carlsbad, CA) and mouse anti-swine IL-10-biotin (IgG1, cat# ASC9109, Invitrogen, Carlsbad, CA), in BD perm-wash solution (50  $\mu$ l), was added to all wells except the secondary antibody control wells and incubated at RT for 15 min. The intracellular primary antibody mix was removed after incubation and cells were washed twice with 100  $\mu$ l BD perm-wash solution. The goat anti-rabbit IgG-AF700 (Alexa Fluor 700, cat# A21038, Invitrogen, Carlsbad, CA) and streptavidin-PerCP-Cy5.5 (cat#551419, BD Pharmingen, San Diego, CA) (50  $\mu$ l) in BD perm-wash solution was added to all wells including the secondary antibody control wells and incubated at RT for 15 min. The cells were washed three times with 100  $\mu$ l BD perm-wash solution. Ultrapure formaldehyde (Polyscience, Warrington, PA) 1% solution in PBS (250  $\mu$ l) was added to all wells and the cells were transferred to flow tubes (Falcon cat # 352008, BD Labware, Franklin Lakes, NJ) and kept in the dark at 4 °C until flow cytometry analysis. The samples were analyzed by the Flow Cytometry Facility, Office of Biotechnology, Iowa State University using FAC-SCanto cytometer (BD Biosciences, San Jose, CA). Data were collected from at least 10,000 live cells of each sample.

### 2.5.4. Flow analysis

FlowJo cell analysis software (Tree Star Inc., Ashland, OR) was used to analyze FCM data. From the total PBMC population, T cell subsets were identified based on the combination of CD4, CD8 and  $\gamma\delta$  TCR expression or lack thereof. The CD4+, CD4+CD8+ (double positive), CD8+,  $\gamma\delta$ + T cells and non T cells (PBMC with no T cell markers) were refer to CD4+CD8- $\gamma\delta$ -, CD4+CD8+ $\gamma\delta$ -, CD4-CD8+ $\gamma\delta$ -, CD4-CD8- $\gamma\delta$ + and CD4-CD8- $\gamma\delta$ -, respectively.

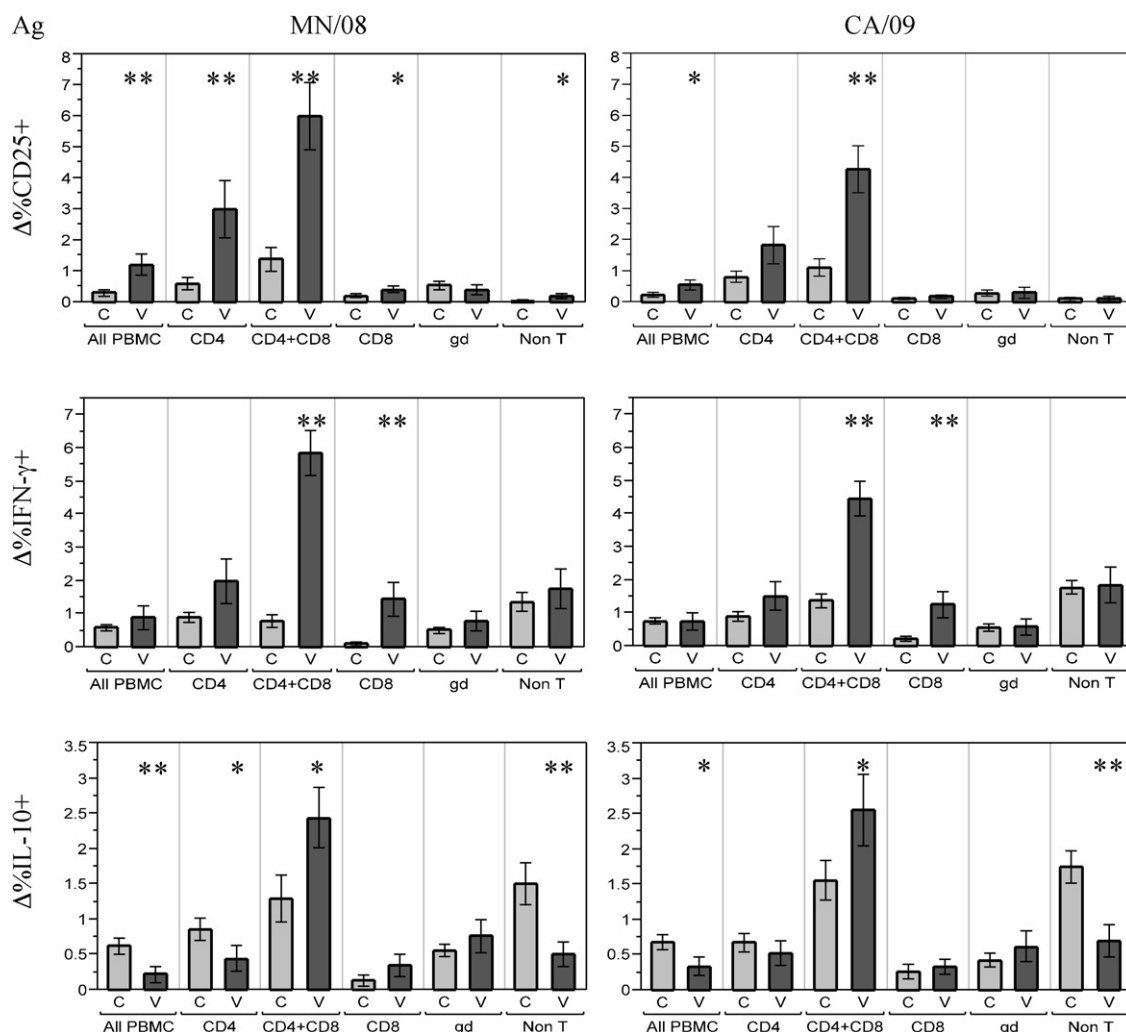
Gates for expression of CD25+, IFN- $\gamma$ + and IL-10+ were set using the non-antigen-stimulated (media only) sample for each T cell subset for each individual pig. The same gates for each parameter of each subset for each pig were applied to the mock and SIV-stimulated samples of the same subset of the same pig. The increase in % positive cells for each parameter was calculated by subtracting % positive cells of non-antigen-stimulated samples from % positive cells of mock-stimulated samples and of SIV-stimulated samples (the average of duplicate wells was used). The net percentage increase was obtained by subtracting the increase of % positive cells of mock-stimulated samples from the increase of % positive cells of SIV-stimulated samples of the same pig. Samples that yielded a net percentage less than 0 were adjusted to 0 before statistical analysis.

### 2.5.5. Statistical analysis

Statistical analysis program JMP 8 (SAS Institute Inc., Cary, NC) software was used to analyze flow data. The significant differences between control and vaccinated groups were evaluated using *t*-test or pooled *t*-test according to their analysis of variances. A probability of *p* < 0.05 was considered statistically significant.

## 3. Results and discussion

Vaccinated pigs demonstrated homologous HI titers ranging from 10 to 160 with a geometric mean  $\pm$  standard error of the mean of  $36 \pm 12$ . There was no detectable



**Fig. 1.** Comparison of net increases in % positive T cell subset responses for CD25, IFN- $\gamma$  and IL-10 between control (C) and vaccinated (V) groups against UV-inactivated homologous MN/08 and heterologous CA/09 viruses (CD4: CD4+CD8- $\gamma\delta$ -, CD4+CD8: CD4+CD8+ $\gamma\delta$ -, CD8: CD4-CD8+ $\gamma\delta$ -,  $\gamma\delta$ : CD4-CD8- $\gamma\delta$ +, Non T: CD4-CD8- $\gamma\delta$ - cells). Significantly different from control group \* $p < 0.05$ , \*\* $p < 0.01$ .

cross-reactivity with heterologous virus. Non-vaccinated control pigs did not develop an HI antibody response to either antigen. The HI results are summarized in Table 1. Humoral responses to SIV vaccines and natural infection have been studied by HI assay (Larsen et al., 2000; Richt et al., 2006; Wesley and Lager, 2006; Van Reeth et al., 2006; Li et al., 2009), ELISA (Larsen et al., 2000; Richt et al., 2006; Li et al., 2009), isotype-specific antibody secreting cell ELISPOT assays (Larsen et al., 2000), Western immunoblot

(Kim et al., 2006) and virus neutralization (VN) tests (Van Reeth et al., 2006; Li et al., 2009). The HI is by far the most commonly used assay. In this study, there was no cross HI reaction between heterologous SIV strains as expected with divergent HA observed between MN/08 and CA/09. Indeed, of the viral structural and non-structural genes, the identity ranged from 91 to 94% for the polymerase genes and 95% for NP and NS, whereas the HA demonstrated only 77% identity. The NA and M genes were of lower identity as well due to the divergence in subtype and genetic lineage, respectively.

Mean net increases of CD25+, IFN- $\gamma$ +, and IL-10+ in PBMC and T cell subsets in response to SIV stimulation are compared in Fig. 1. Pigs vaccinated with inactivated whole virus with adjuvant had significantly higher net %CD25+ in all T cell subsets with the exception of  $\gamma\delta$ + T cells in response to homologous recall antigen. In contrast, PBMC from vaccinated pigs cross-reacted to heterologous recall antigen only in the CD4+CD8+ subset. The net %IFN- $\gamma$ + responses of the vaccinated pigs were significantly higher

**Table 1**

Serum geometric mean HI titers  $\pm$  standard errors of mean against UV-inactivated homologous MN/08 and heterologous CA/09 viruses.

Antigen	Group	
	Control	Vaccinated
Homologous MN/08	0.0 $\pm$ 0.0	36 $\pm$ 12**
Heterologous CA/09	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

\*\* Significantly higher than control group and heterologous antigen,  $p < 0.01$ .

than the control group in CD4+CD8+ and CD8+ for both homologous and heterologous recall antigens. Interestingly, the net %IL-10+ responses of the vaccinated pigs were significantly lower than the control group in the CD4+ subset against homologous antigen and the non T cells of both antigens, while the CD4+CD8+ subset had significantly higher net %IL-10+ responses to both antigens. There were no significant changes in the expression of CD25, IFN- $\gamma$  or IL-10 in the  $\gamma\delta$ + population between control pigs and vaccinated pigs after restimulation with either virus type.

Cellular responses to SIV have been less commonly reported and studied only by T cell proliferation (Kitikoon et al., 2006, 2009a, 2009b) and IFN- $\gamma$  ELISPOT assays (Larsen et al., 2000). A report in pigs showed a significant increase in SIV specific CD4+CD8+ proliferation, but no significant changes in other T cell subsets, when stimulated with either the homologous or heterologous inactivated viruses (Kitikoon et al., 2006). The authors concluded that the vaccine was able to induce a systemic SIV-specific memory T cell response. We observed similar results by MP-FCM in that the inactivated vaccine could prime the CD4+CD8+ T cell subset, a population of T cells that have been demonstrated to be memory T cells in swine (Zuckermann and Husmann, 1996; Charerntantanakul and Roth, 2007). Although we did not measure antigen-specific proliferation, which is often used for evaluating antigen-specific recall responses, we did evaluate changes in expression of intracellular IFN- $\gamma$  (Th1 cytokine) and IL-10 (Th2 cytokine), both of which are produced in response to specific recall antigens. In addition, we evaluated antigen-specific changes in the expression of CD25, the high affinity  $\alpha$  chain of the IL-2 receptor present on activated T cells on each T cell subset. The MP-FCM results from our study detected increased expression of all 3 parameters in the CD4+CD8+ T cell of vaccinated pigs in response to both heterologous and homologous recall antigens. In addition, our results showed a decrease in IL-10+ cells in CD4+ T cells and non T cells. The decrease in IL-10+ CD4+T cells was only significant in response to homologous antigen. Without examining T cell subset identification, the results from total PBMC would indicate a reduction in overall IL-10 expression. Thus, T cell subset identification is important when evaluating the percentage of cells producing cytokine in response to recall antigen. The reason for the decrease in IL-10+ T cell subsets is unknown. IFN- $\gamma$  has been shown to inhibit Th2 cytokine production (Chomarat et al., 1993). These data suggest that IFN- $\gamma$  may inhibit IL-10 production in CD4+ T cells and non T cells, but not in CD4+CD8+ T cells in pigs. According to porcine T cell subset phenotypes described by Gerner et al. (2009), the CD8+ T cell phenotype in this study includes natural killer (NK) cells which may explain the ability of the inactivated vaccine to prime the CD8+ T cells as evidenced by a significant increase in the percentage of IFN- $\gamma$ + cells in response to both recall antigens. A combination of IL-12 and IL-18 has been shown to stimulate NK cells for enhanced IFN- $\gamma$  production (Gerner et al., 2009). Perhaps these cytokines produced by CD4+ T cells stimulated IFN- $\gamma$  production by CD8+ NK cells. On the other hand, the UV-inactivated recall viruses used in this study may be able to enter the cells and uncoat in the cytoplasm. The translation mechanism may not be completely

blocked. In this case, it is possible that the translated viral antigens are presented on MHC I to CD8+ T cells. There is also the possibility of cross-presentation of viral proteins contained in the vaccine preparation onto MHC I through the exogenous pathway (Murphy et al., 2007).

The A/SW/MN/02011/08 H1N2 SIV vaccine used in this study was demonstrated to effectively prime both humoral and cellular immune responses. However, cross reactivity to the UV-inactivated heterologous pandemic H1N1 CA/09 recall virus was observed in cellular but not in the HI humoral responses. The cellular cross reactivity to UV-inactivated heterologous virus measured by the MP-FCM suggests that the assay is sensitive and likely detects a wider range of viral epitopes than the HI assay. This suggests that inactivated SIV vaccines may provide some protection against heterologous infection, even when cross-reactivity is not observed in the HI assay. It will be important to characterize the homologous and heterologous T cell subset responses using live recall viruses to gain a more complete understanding.

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