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# Pandemic and seasonal H1N1 influenza hemagglutinin-specific T cell responses elicited by seasonal influenza vaccination

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ABSTRACT

Understanding whether seasonal influenza vaccines can elicit antibody and T cell responses against the 2009 pandemic H1N1 strain is important. We compared T cell and antibody responses elicited by trivalent inactivated influenza vaccine (TIV) and live attenuated influenza vaccine (LAIV) in healthy adults. Both vaccines boosted pre-existing T cells to the seasonal and pandemic hemagglutinin (HA) but responses were significantly greater following immunization with LAIV. Antibody titers were significantly boosted only by TIV. The relationship between antibody and T cell responses and the effect of the magnitude of pre-existing immunity on vaccine-induced responses were also evaluated. Cross reactive T cell responses to the pandemic H1N1 HA existed among the cohort before the circulation of the virus to varying degrees and these responses were boosted by seasonal vaccination.



## 1. Introduction

Seasonal influenza epidemics are estimated to cause an average of 36,000 deaths annually in the United States and are a source of considerable morbidity and mortality globally [1–3]. Unlike seasonal epidemics, influenza pandemics such as the 1918 pandemic are estimated to have caused over 40 million deaths worldwide [4]. Centers for Disease Control and Prevention (CDC) estimates that the newly emergent 2009 pandemic H1N1 may have caused over 86 million infections and 17 thousand deaths in the United States alone [5].

Two distinct influenza vaccines are currently licensed for use in humans; an inactivated vaccine and a live attenuated vaccine. Both licensed influenza vaccines include antigenic components from three type-specific influenza strains chosen annually to match dominant globally circulating influenza viruses. Inactivated and subvirion influenza vaccines were first tested in humans in the early 1940s [6] and their use has continued to the present. Trivalent inactivated influenza vaccine (TIV) is delivered intramuscularly and is standardized based on hemagglutinin (HA) content. The other vaccine, a live attenuated influenza vaccine (LAIV), was licensed for human use in 2003 and consists of replicating pseudotyped viruses that replace seasonally matched influenza HA and neuraminidase (NA) genes onto a temperature sensitive, laboratory adapted influenza genetic backbone [7]. LAIV is a mucosal vaccine delivered intranasally and its immunogenicity is likely dependent on the ability of the vaccine-virus to replicate in the host. TIV and LAIV appear to provide varying levels of protection depending on the age of the population and the antigenic match to the circulating epidemic influenza strains [8–10].

Current consensus on the correlates of immunity to influenza suggests antibody-mediated protection reduces both the incidence and severity of influenza disease [6]. However, such antibody responses are mainly subtype-specific, and hence are unlikely to protect vaccinees against major antigenic shifts characteristic of influenza pandemics. In fact, a number of studies have failed to document the existence of significant cross-reactive antibodies to the novel pandemic H1N1 except among the elderly who may have previously been exposed to antigenically related viruses [11]. Similarly, while seasonal influenza vaccination does not induce significant cross reactive antibodies to the novel pandemic H1N1, recent evidence strongly suggests that such seasonal vaccination could provide partial protection against the novel pandemic influenza of 2009 [12,13]. This suggests that vaccination may have induced cross-reactive T cell responses that provided this protection. It is possible that T cell responses elicited against conserved viral epitopes results in long-lived, subtype-independent immunity that could provide protection from influenza infection or reduce severity of influenza disease. In fact, accumulating evidence suggests cellular immunity could be an alternate correlate of protection against influenza [14–17].

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Emerging evidence suggests that HA is a significant component of not only antibody but also influenza T cell responses [16]. However, our current understanding of the T cell immunity to this important antigen is limited. While recent reports have documented the existence of cross-reactive CD4+ T cell responses to potential pandemic H5N1 HA antigens, whether similar cross-reactive CD4+ T cell also exist against the novel 2009 pandemic H1N1 HA is currently not clear. It is also unclear if seasonal H1N1 vaccination can boost such cross-reactive T cell responses. Elucidation of T cell responses to seasonal and pandemic H1N1 HA following seasonal influenza vaccination can help in the design of vaccines that enhance cross protection.

## 2. Subjects and methods

### 2.1. Human subjects and sample collection

The study was performed at Cincinnati Children's Hospital Medical Center following appropriate Institutional Review Board (IRB) and Institutional Biosafety Committee approvals. All samples were acquired during the fall of 2007, before seasonal influenza circulation in the community and well before the emergence of the pandemic H1N1 in April 2009. A total of 30 healthy subjects between the ages of 18 and 49 years were recruited and received vaccine between October and November of 2007. Fifteen subjects were assigned to receive the licensed live attenuated influenza vaccine containing the 2007–2008 seasonal influenza antigens intranasally (FluMist™; MedImmune Inc) and an additional 15 were assigned to receive the trivalent inactivated vaccine (Fluarix™; GlaxoSmithKline) containing the 2007–2008 seasonal vaccine antigenic components intramuscularly. The H1N1 component of the 2007–2008 seasonal TIV and LAIV vaccines incorporated the A/Solomon Islands/3/2006 (H1N1)-like virus.

Subjects who had an acute influenza like illness (ILI), or laboratory diagnosed influenza (LDI) within the preceding 3 months were excluded. Individuals who had received any other live or inactivated vaccine in the preceding 30 days, who were pregnant or had given birth within the preceding two months, diagnosed with cancer, receiving immunomodulatory therapy, or otherwise suffering from chronic disease were also excluded. Other exclusion criteria included known allergy to any component of the vaccines; or a history of Guillain–Barré syndrome. Blood specimens were collected prevaccination on the day of vaccination and 2 weeks postvaccination in Vaccutainer heparin tubes for PBMC isolation and parallel SST tubes for sera collection (Beckton Dickenson).

### 2.2. ELISPOT assay

Peripheral blood mononuclear cells (PBMCs) were purified following Ficoll Hypaque gradient centrifugation and cryopreserved in liquid nitrogen using endotoxin free 90% fetal bovine serum and 10% DMSO. PBMCs were stimulated for seven days in the presence of media or overlapping HA peptide pools derived from either the seasonal or the pandemic H1N1. All PBMC cultures received 20 Units/ml human recombinant IL-2 on day 3 and 6 (Hoffmann-La Roche). The seasonal strain was H1N1 A/New Caledonia/20/99 which shares 98% sequence identity with the Solomon Islands/3/2006 (H1N1) found in the 2007–2008 vaccine. Pandemic HA was represented by the reference pandemic strain of H1N1 A/California/04/2009. Peptides were 16–17 amino acid long with 11–12 amino acid overlaps (H1N1 A/New Caledonia/20/99; BEI Resources: H1N1 A/California/04/2009; custom synthesis; Mimotopes). These overlapping peptides spanned the entirety of the HA protein sequence (565aa seasonal and 566aa pandemic) in 94 peptides and were split into two equal pools of 47 peptides each for PBMC stimulations. The two overlapping pools spanned amino acids 1–290 and 280–565/566 of the HA sequences covering the first and second half of the proteins. Total HA responses were expressed as the sum of the IFN-γ SFU elicited by these two contiguous overlapping pools. The pandemic HA sequence when aligned to the seasonal HA (Clustal W alignment; Lasergene™, Madison, WI) revealed 97 substitutions and a single insertion at position 147; accordingly, overlapping pandemic HA peptides that span this site incorporate an added residue. Due to the dispersion of the variant residues in the pandemic HA sequence, only 19 of the 94 peptides were completely conserved between the seasonal and pandemic antigens, with the remaining 75 peptides incorporating one or more substitutions or insertion. Optimal concentrations of peptides were first determined by flow cytometry. Using the optimal concentration of 0.5 μg/ml, >96% of the total responses observed among 15 subjects were from CD4+ T cells with only 3 subjects achieving 2–4% responses from the CD8+ compartment. Thus as reported earlier [18], these longer peptides preferentially stimulated CD4+ T cells.

The secretion of interferon gamma (IFN-γ) was detected by ELISPOT assays. Multiscreen 96-well plates (Millipore) were coated overnight at 4 °C with 1 μg/ml anti-human IFN-γ capture antibody (Mabtech), washed with PBS and blocked for 4 h with complete RPMI medium (Invitrogen). Cultured lymphocytes were added at $5 \times 10^4$ cells/well in triplicate and stimulated with peptide pools or media for 18 h at 37 °C.

Phytohemagglutinin, PHA (Sigma) at 5 μg/ml was used as a positive control. Plates were washed with PBS and incubated with antihuman IFN-γ mAb 7B6-1-ALP (Mabtech) for 2 h at 37 °C and washed again with PBS before chromogenic development with NBT/BCIP. Spot forming units (SFU) were enumerated using an automated ELISPOT reader (CTL Technologies) and mean SFU was calculated for each stimulation after subtracting values from negative control wells. Data are presented as mean SFU per $10^6$ PBMCs using standard procedures we and others have described earlier [19,20].

### 2.3. HAI assays

Sera were stored at −80 °C, thawed and treated overnight at 37 °C with RDE (receptor-destroying enzyme) derived from Vibrio cholerae (Denka-Seiken), heat inactivated at 56 °C and utilized in standard HAI assay. Seasonal H1N1 A/Solomon Islands/3/2006 and pandemic H1N1 A/California/04/2009 antigens were acquired from Centers for Disease Control and Prevention (CDC), courtesy of Dr. Alex Klimov and Dr. Xiyan Xu; and Protein Sciences Corp. Four units of hemagglutinin were incubated with serially diluted RDE treated sera for 30 min at ambient temperature. Turkey red blood cells (Viromed, CT) were added at 0.5% to the antigen-sera wells and incubated at room temperature for 30 min to determine agglutination inhibiting titers. Sera with titers <4 were assigned a titer of 2.

### 2.4. Statistical analysis

Matched T cell responses against the seasonal and pandemic HA antigens coexistent within the same donor were tested by two-tailed Wilcoxon signed rank test. Comparison of responses elicited by the TIV vaccinees to those from LAIV vaccinees, and responses elicited by low and high baseline immune responses were tested by two-tailed Mann–Whitney test. The relationship between cell-mediated immune responses and hemagglutinin inhibition assays and the relationship between pandemic and seasonal T cell responses were tested by nonparametric two-tailed Spearman correlation. $P$ values of $\leq 0.05$ (two-tailed) were considered significant (GraphPad Prism, Version 5.01).