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Qualitative Differences in T cell responses to Live, Attenuated and Inactivated Influenza Vaccines

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Abstract

Annual epidemics of influenza cause considerable morbidity and mortality. Trivalent inactivated vaccine (TIV) and live, attenuated influenza vaccine (LAIV) are licensed in the United States, and both are effective in preventing disease in persons younger than 49. Serum hemagglutination inhibition (HI) titers correlate with TIV but not LAIV efficacy, suggesting that additional effector mechanisms are induced to the live, attenuated vaccine and play an important role in protection against disease. For this reason there is a need to identify surrogate markers of LAIV efficacy that are easily measured in robust assays. We have compared the immunogenicity of TIV and LAIV in a small clinical study (16 age-matched volunteers in each vaccine group) by measuring serologic responses using traditional HI and NA inhibition assays as well as a sensitive cell-based neutralization assay. In addition, we evaluated cellular responses by measuring the quantity and quality of antigen-specific $CD4^+$ and $CD8^+$ T cells following vaccination. The quality of the $CD4^+$ T cell response was different for each vaccination group, with $CD4^+$ T cell proliferation and increased secretion of IFN-γ characteristic of responses following immunization with LAIV, while antigen-specific T cells that secreted IL-5 were more frequently measured from TIV recipients. Our results suggest that sensitive, serologic assays with broad specificity, together with $CD4^+$ T cell proliferation and IFN-γ secretion provide a more complete measure of the immunogenicity of LAIV in adults, and could be used to enhance the identification of vaccine responders.

Keywords: Influenza; Vaccine; Serology; T cell responses

Abbreviations: TIV: Trivalent inactivated vaccine; LAIV: Live, attenuated influenza vaccine; HI: Hemagglutination Inhibition; NI: Neuraminidase inhibition; AVINA: Accelerated viral inhibition assay with NA as read-out

Introduction

Influenza viruses cause annual epidemics during winter months, with substantial respiratory illness and mortality worldwide, particularly in the elderly and very young [1]. The extent of the epidemic is impacted by viral fitness and population immunity, with virus replication controlled by HA and NA-specific antibodies that block virus entry and release, and influenza-specific $CD4^+$ or $CD8^+$ T cells that secrete cytokines with antiviral properties, or kill infected cells [2]. Influenza has a segmented RNA genome, and consequently lacks genetic proof-reading ability. This allows selection of variants with altered antigenic structures, enabling escape from prior immunity. The licensed trivalent influenza vaccines are therefore reformulated annually to ensure inclusion of the antigenically appropriate influenza A (H1N1 and H3N2) and B virus [3].

Antibody responses to hemagglutinin (HA) as measured by HA inhibition (HI) assay, correlate with the efficacy of trivalent inactivated influenza vaccines (TIV), but the host responses that contribute to immunity in response to live, attenuated influenza vaccine (LAIV) are not well characterized. HI titers after LAIV are often not robust [4], and therefore results from this assay are likely to underestimate the vaccine's immunogenicity. Additional antibody and T cell mechanisms are likely to contribute to protection, and may provide alternative ways to evaluate vaccine immunogenicity and efficacy. There is some evidence that the correlates of broader protection include the presence of IgA in the upper respiratory tract and antibodies with specificity for viral neuraminidase (NA) [5]. Clinical studies have also shown a correlation between IFN-γ secreting T cells and protection against disease [6], and this effector mechanism may contribute to the protection against antigenically-drifted viruses observed in children [7].

In April 2009, a swine-origin H1N1 virus emerged in humans that was antigenically distinct from other viruses previously circulating, resulting in efforts to rapidly manufacture and distribute a vaccine with matching HA and NA antigens. Both monovalent inactivated and live, attenuated vaccines were licensed and available by October 2009, and this strain was subsequently included as the H1N1 component of seasonal trivalent influenza vaccines. Clinical studies suggest that the pandemic inactivated vaccine was effective in reducing infection in older children and younger adults, while the live, attenuated vaccine had lower efficacy in adults [8]. A recent meta-analysis suggests seasonal influenza vaccine effectiveness varies between studies, and should be improved [9]. Although HI titers ≥40 are considered protective in adults, an HI titer of 85 (at time of exposure), correlates with protection of 50% of 6-72 month old children immunized with an inactivated vaccine [10]. Even the lower titer is often not achieved in young children vaccinated with LAIV, and yet this vaccine is effective

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in this population group [11]. Additional serologic or cellular measures of an immune response that correlate with protection against disease would be valuable in assessing the immunogenicity of LAIV and its potential effectiveness.

Animal models demonstrate various ways in which $CD4^+$ T cells contribute to protection against influenza [12]. While traditionally classified as "helper" cells required to support antibody isotype switching and antibody secretion, influenza-specific $CD4^+$ T cells may also contribute to limiting virus replication by secretion of cytokines or cytolytic activity. The interactions and soluble factors elicited during initiation of the response are different for TIV and LAIV and would be expected to induce distinct $CD4^+$ T cell types following immunization that may dictate the quality and quantity of the influenza-specific antibody response. This hypothesis is supported by findings of influenza studies in which distinct cytokines are elicited following different immunization regimes [13,14], with antibody responses that are in line with the paradigm that murine IgG1 responses are dependent on IL4-producing $CD4^+$ T cells, and IFN-γ supports IgG2a responses [15]. A systems biology approach to compare human responses to LAIV and TIV showed increased amounts of mRNA for antiviral molecules in response to the infectious but not inactivated vaccine in circulating cells, and increased amounts of the chemokine IP-10 in plasma of individuals vaccinated with TIV but not LAIV recipients [16]. The antibody response was more robust following TIV, and therefore it is not surprising that this group had greater numbers of antibody secreting cells with upregulation of genes associated with antibody production; in contrast, the genes upregulated in response to LAIV were indicative of T cell, NK cell and monocyte activation. The induction of cellular responses by LAIV is confirmed in clinical studies: for example, increased numbers of influenza-specific $CD4^+$ and $CD8^+$ T cell responses can be measured in young children vaccinated and boosted with LAIV but not TIV [17]. A difference in the magnitude of the T cell response following LAIV and TIV is difficult to identify in adults, possibly due to the presence of a population of cross-reactive memory T cells induced by previous infection or vaccination [18].

We hypothesized that the $CD4^+$ T cell responses following TIV and LAIV are qualitatively distinct, and may consequently need to be measured in different ways when used as an indicator of vaccine immunogenicity. In our evaluation, we measured antibody and T cell responses of age-matched healthy adults vaccinated with either TIV or LAIV in a small clinical study. Serum and peripheral blood mononuclear cells (PBMC) were collected from these vaccinees immediately before and 4 weeks after routine seasonal vaccination. Since immunodominant T cell epitopes vary significantly depending on HLA type, PBMC were stimulated by addition of whole virus to the cultures and responses were quantified by measuring T cell proliferation and cytokines secreted into the supernatant. Our results confirm the work of others showing greater HI responses after TIV than LAIV, and greater $CD4^+$ T cell proliferation following LAIV than TIV [16]. In addition, our results show that the cellular responses after vaccination with LAIV and TIV are distinct: a greater number of LAIV recipients had increased IFN-γ secretion, whereas IL-5 secretion was more frequently increased in TIV-immunized adults.

Materials and Methods

Virus preparation

The following influenza viruses were grown in 10-day old embryonated chicken eggs: Viruses corresponding to the 2006/07 vaccine: A/New Caledonia/20/99 (H1N1) (A/NC/99), A/Wisconsin/67/2005 X161B (H3N2) (A/WI/05), B/Malaysia/2506/04 (BM/04), and viruses used for NA inhibition assays: $H6N1_{NC/99}$, and $H6N1_{WI/05}$. The latter 2 viruses were generated by reverse genetics as previously described [19].

Clinical study design

The clinical study population included 32 healthy consenting adults between the ages of 18 and 49 that were enrolled into the study prior to the administration of the seasonal influenza vaccine at Brooke Army Medical Center (BAMC) in November 2006. The study was approved by the BAMC Institutional Review Board, and de-identified samples were used in serologic and cellular assays in a protocol approved by the FDA Research Involving Human Subjects Committee. Volunteers were age-matched between groups receiving LAIV and TIV. Individuals with immunodeficiency or active immunosuppressive treatments (including systemic corticosteroids) were excluded from the study. Any contraindication to receiving either the TIV or LAIV was an exclusion criteria. The demographics of subjects in each vaccination group are shown in Table 1. Each volunteer donated a blood and nasal wash sample immediately before and approximately 28 days after receiving either trivalent live, attenuated influenza vaccine (Flumist, MedImmune) or trivalent inactivated vaccine (Fluzone, Sanofi-Pasteur). Blood was drawn into BD Vacutainer® CPT and BD Vacutainer® serum collection tubes. The PBMC were washed and aliquots frozen in 10% DMSO in liquid N2; serum was aliquoted and stored at -20 °C.

Antibody assays

Hemagglutination Inhibition (HI) assay: HI titers were measured as previously described [20]. Briefly, sera were treated with receptor destroying enzyme and then heat-inactivated. Non-specific agglutinins were removed by adsorption with packed washed chicken red blood cells (RBC). Serial dilutions were made in 25 µl PBS and an equal volume of PBS containing 4 HAU of virus added to each well. After 30 minute incubation, 0.5% washed chicken RBC were added, the contents of the wells mixed and then incubated at room temperature for 45 minutes before recording agglutination. The inverse of the last dilution that resulted in inhibition of agglutination was recorded as the titer.

Neuraminidase inhibition assay: Titration of serum NI antibodies was performed by analyzing NA activity of the HA-mismatched reassortant viruses in a 96-well plate format of the conventional thiobarbituric acid assay [19]. Briefly, serum specimens were serially diluted in PBS across wells of 96-well PCR plates. Virus suspended to

Table 1: Demographics of study participants.

	Vaccine group	
	LAIV	TIV
Number	16	16
Mean age (range)	32.9 (18-49)	32.5 (22-47)
Percent male	38	69
Ethnicity		
White	8/16	9/16
Hispanic	6/16	5/16
Asian	2/16	2/16
Seasonal allergies	1/16	1/16

a standard NA activity level was added in equal volume, followed by fetuin. After overnight incubation, liberated sialic acid was detected by chemical reactions which produce a chromophore measured at OD_{550}. NI endpoint titers were determined as the reciprocal of the highest serum dilution that inhibited NA signal by ≥ 50%.

AVINA Neutralization assay: An accelerated viral inhibition assay with NA as read-out (AVINA assay) was used to measure titers as previously described [20]. Briefly, MDCK cells were washed in serum-free medium (EMEM containing glutamine, penicillin and streptomycin) and 50 µl of a 8 x 10^5/ml cell suspension placed in wells of flat-bottomed 96-well plates. The cells were incubated overnight at 37 °C in 5% CO_2. The next day, sera were heat inactivated for 30 min at 56 °C and serial two-fold dilutions made in serum-free EMEM. Triplicate dilution wells were made of each sample, with paired sera (pre and post vaccination) diluted on the same plate. Virus was added to EMEM containing 3% BSA and TPCK-treated trypsin (5 µg/ml) so that 70 µl, the volume added to each well, contained virus at a multiplicity of infection (MOI) of ~0.01 (400 $TCID_{50}$). Virus and sera were mixed by shaking the plate for 5 min before 100 µl was transferred to the MDCK containing plates. The plates were incubated for 20 hr at 37 °C in 5% CO_2. NA substrate, 20 µM methyl-umbelliferyl-N-acetyl-neuraminic acid (MU-NANA), was then added (75 µl per well). After 1 hr incubation at 37 °C, stop solution (0.1 M glycine, pH 10.7 in 25% EtOH) was added to each well (100 µl/well) and fluorescence read on a Victor V plate reader (Perkin Elmer) with excitation and emission filters of 355 nm and 460 nm respectively.

T cell analyses

$CD4^+$ T cell analysis: Responses of PBMC obtained before and 4 weeks after vaccination were always compared in the same assay. Frozen cells from each time point were thawed, washed, and rested for 30 minutes in RPMI containing antibiotics (Pen/Strep), non-essential amino acids, sodium pyruvate, β-mercaptoethanol, L-glutamine, and 10% human serum (complete RPMI). Cells were labeled with CFSE by adding 10 µM to a 100 µl suspension of 3-5x10^6 cells. The cells were incubated for 10 min at 37 °C before adding 1ml cold complete RPMI. The cells were washed, resuspended at 1 x 10^7 cells/ml and 100 µl distributed into wells of a 96-well flat bottom plate that had been coated the previous day with 50 µl of virus suspension (H1N1, H3N2, or B) at 50 HAU/ml. The viruses used for stimulation, A/NC/99 (H1N1), A/WI/05 (H3N2) and BM/04, were the same strains as included in the 2006/07 influenza vaccines administered to volunteers in this study. Wells containing complete RPMI were used as negative control; wells containing tetanus toxoid (2 µg/ml) from *Clostridium tetani* (Calbiochem, UK) and Staphylococcus enterotoxin type B (SEB, Sigma Aldrich, St Louis, MO) at 1µg/ml were used as positive controls. All cultures were run in triplicate. Plates were incubated for 3 days at 37°C in 5% CO_2 before 100 µl supernatants were collected for cytokine analysis. An equal volume of complete media was added back to each well and on day 5 of the culture, 0.2 ng/mL IL-2 was added to each well. On day 7 of culture, cells were resuspended and then transferred to a 96-well U-bottom plate for staining with relevant antibodies.

$CD8^+$ T cell analysis: $CD8^+$ T cells were most efficiently stimulated by dendritic cells (DC) generated *in vitro* from monocytes. To generate the DC, monocytes were enriched from PBMCs using CD14 microbeads (Miltenyi Biotec, Auburn, CA) and then cultured for 6-7 days with IL-4 (50 ng/ml) and GM-CSF (100 ng/ml) in RPMI with 10% FCS. These DCs were pulsed with medium (negative control), a mixture of M and NP influenza peptides (1 µg/ml) or a mixture of CMV, EBV and influenza (CEF) peptides (positive control) (1 µg/ml) in serum-free RPMI for 1 hr in a 37°C water bath and then washed twice. Influenza peptides were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository (NIAID, NIH), and the CEF peptide mixture was obtained from the AIDS Research Resource (NIAID, NIH). The syngeneic $CD14^-$ fraction was used as the source of T cells and therefore cryopreserved prior to use. These were thawed and labeled with 1 µM CFSE as described above and then plated at 0.5 to 1 x 10^6/well in 96-well flat-bottom plates in complete RPMI with 10% human serum and 0.1 ng/ml IL-2. Antigen-pulsed DCs were added to the wells at a DC/T ratio of 1:20. The cocultures were incubated at 37°C for 7 days. Samples were then restimulated for 6 hr with or without peptide (1µg/ml) in the presence of 1µg/ml αCD28 and αCD49d (BD Biosciences, Mountain View, CA) and brefeldin A (10 µg/ml). The cells were fixed, permeabilized and stained with a combination of fluorochrome-conjugated antibodies: CD8 Pacific Blue, IL-2 APC, IFN-γ Alexafluor 700, TNF-α PE-Cy7, Granzyme B Alexafluor 700, or CD107a APC (BD Biosciences). Cells were analyzed on a FACSCanto II (BD Biosciences) using FlowJo software (TreeStar).

Cytokine analysis

Cytokine analysis was performed on all samples using the Meso Scale Discovery (MSD; Gaithersburg, MD) electrochemiluminescence platform. A multiplex 96-well plate format was used, with simultaneous measurement of IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, IFNγ, and TNFα. All reagents and pre-coated plates were purchased from MSD, and the manufacturer's protocol was followed. Briefly, all reagents were brought to room temperature prior to use, and all incubations performed at room temperature with shaking. Culture supernatants (25 µl) from PBMC that had been restimulated 3 days previously and calibrators were added to the plate and incubated for 2 hrs. After washing the plates with PBS-0.05% Tween-20, ruthenium-labeled detection antibody was added and the plates incubated for an additional 2 hours. A final wash was performed, followed by the addition of read buffer. Detection was initiated by applying voltage to electrodes located on the bottom of the plate. Intensity of light emission was captured on the MSD Sector Imager 2400. Cytokine concentrations were determined using a curve fit model with software provided with the instrument.

Statistical analysis

Geometric mean titers were calculated for all assays in which end-point titers were assigned and statistical differences before and after vaccination determined by paired t test; differences between groups were determined by Mann-Whitney test, with statistical significance, $p \leq 0.05$.

Results and Discussion

Comparison of antibody responses following receipt of live and inactivated vaccines

Geometric mean titers (GMTs) of sera from individuals in each group are shown in Table 2 for assays conducted with the homologous antigens included in the vaccine (NC/99, WI/05 and BM/04), together with NA inhibition (NI) titers against NA of NC/99, WI/05. The cohort was not prescreened, and considering this study was performed

Table 2: Serologic responses following LAIV and TIV. Geometric mean titers of HI, NI and neutralizing (AVINA) antibodies are shown for all individuals in each vaccine group (n=16) before and after vaccination (pre and post), and percent of individuals with a significant increase in titer (response rate) following immunization with LAIV and TIV.

		Virus strain used in assay								
		A/NC/99 (H1N1)			A/WI/05 (H3N2)			BM/04		
Test[a]	Vaccine	pre	post	Response rate[b]	pre	post	Response rate	pre	post	Response rate
HI	LAIV	54	80	6	57	77	13	11	12	0
	TIV	135	160	19	104	258	31	24	38	19
NI	LAIV	11	14	31[c]	11	13	38[c]	ND	ND	ND
	TIV	10	13	38[c]	14	19	44[c]	ND	ND	ND
Neutralization	LAIV	197	234	56	133	150	50	128	161	38
	TIV	263	363	75	101	223	94	182	382	81

[a]Serologic tests are described in Methods and Materials: the HI assay follows a standard protocol; the NI assay followed a miniaturized thiobarbituric acid method to quantify sialic acid; the neutralization assay followed a novel "AVINA" protocol in which NA activity is used as an end-point.

[b]Response rate is the percent of individuals in each group that had a statistically significant increase in titer: for HI assays this is a 4-fold increase; for NI this is a 2-fold increase; for neutralizing antibodies this is a statistically significant increase in the IC50 determined by non-linear regression.

[c]Previously reported [20].

in a group that is offered seasonal vaccination each year, it was not surprising that baseline HI titers to H1N1 and H3N2 components were relatively high, with 'seroprotective' titers (≥40) in the majority of individuals. Individuals in this study had low pre-existing titers to the B component, possibly reflecting low circulation of this virus during the preceding influenza season and use of an antigenically-distinct strain in the previous season's vaccine (B/Shanghai/361/2002 of the B/Yamagata lineage was used in 2005/06 season while the individuals in this study were immunized in September 2006 with a vaccine containing BM/04 of the B/Victoria lineage).

A four-fold increase in HI titer is traditionally used to evaluate vaccine immunogenicity. The problem in using this test is demonstrated in several ways in this small study: (a) there was no significant increase in HI GMT against the H1N1 component of the inactivated vaccine (p=0.12). This is more likely to reflect relatively high baseline titers that are not easily increased [21,22], than poor vaccine immunogenicity; (b) while the LAIV group had a significant increase in their HI GMT to the H1N1 component (p=0.01), and there was a trend toward significant increase in GMT against the H3N2 component (p=0.06), there was no significant increase in HI titers to the B component (p=0.35). Despite an increase in the GMTs, there were very few individuals immunized with LAIV with a 4-fold increase in HI titer to any of the 3 vaccine components, and this response rate was less than observed for TIV recipients (Table 2). This is consistent with other reports that show HI responses are more easily observed in response to TIV than LAIV [16,22,23].

Human challenge studies demonstrate increases in NI titers correlate with protection [24], and therefore we included this measure in our study. Some GMTs shown in Table 2 were previously reported [20], but are repeated here to emphasize that the response rate measured by NI assay was not significantly different between vaccine types.

Response rates were greatest when antibody titers were measured by an AVINA neutralization assay, an assay that has excellent sensitivity and includes detection of functional antibodies with specificity for both HA and NA [20]. Even with this assay, there were significantly fewer responders to each of the 3 virus strains in LAIV than TIV (Table 2).

Comparison of CD8$^+$ T cell responses following receipt of live and inactivated vaccines

The number of cells available limited the number of CD8$^+$ T cell assays that could be performed. Consequently, proliferation of CD8$^+$ T cells isolated 28 days post-vaccination was compared with cells obtained prior to vaccination for 10 individuals vaccinated with LAIV and 6 individuals vaccinated with TIV. Compared to proliferation in the control wells that did not contain antigen, neither vaccine group has increased CD8$^+$ T cell proliferation on day 28 following vaccination. There were very few antigen-specific CFSE^{dim} CD8$^+$ T cells in cultures from 4 LAIV recipients and therefore responses were compared between the remaining 6 individuals vaccinated with LAIV and 6 individuals vaccinated with TIV. The median percentage of cytokine-expressing antigen-specific CD8$^+$ T cells, together with the minimum, maximum and first and third quartile, is shown for each group in Figure 1. There were no significant differences between the percent of cells expressing IL-2, IFN-γ, TNF-α, CD107 or granzyme b, 28 days after vaccination with LAIV and TIV. It was not expected that TIV would stimulate CD8$^+$ T cell responses as inactivated antigens do not efficiently prime CD8$^+$ T cells, and therefore the stimulation observed in these cultures is more likely to reflect stimulation of memory cells generated in response during a prior infection, rather than vaccination. Our data suggest that vaccination of adults with LAIV and TIV does not result in quantitative or qualitative differences in the CD8$^+$ T cell response measured 4 weeks after vaccination. This result is supported by a larger study comparing responses to LAIV and TIV [25]. However, this latter study showed upregulation of CD27

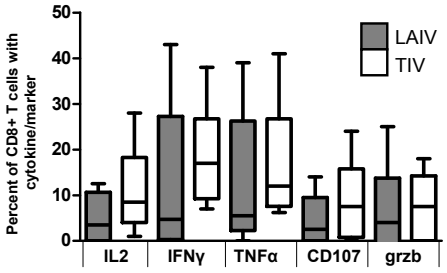

Figure 1: Percent of CD8⁺ T cells from individuals vaccinated with LAIV or TIV expressing IL2, IFN-γ, TNF-α, CD107a or Granzyme B (grzb). The T cell cultures were stimulated with monocyte-derived DC that had been loaded with a pool of influenza peptides as described in Materials and Methods. The box and whisker plot shows the median percent of CD8⁺ T cells expressing cytokine/antigen for each group within the box; the ends of the box representing the 1st and 3rd quartiles, and the minimum and maximum values. There were no statistical differences between the medians for each marker before and after vaccination with LAIV and TIV (Mann-Whitney, p>0.05).

on IFN-γ+CD8⁺ T cells 10 days after TIV vaccination, demonstrating the importance of examining differences in CD8⁺ T cells at early time points after vaccination.

Comparison of CD4⁺ T cell responses following receipt of live and inactivated vaccines

To determine whether influenza-specific CD4⁺ T cell responses differ after immunization with LAIV and TIV, and whether the CD4⁺ T cell response can be used as an indicator of LAIV immunogenicity, we evaluated CD4⁺ T cell proliferation, determined whether these cells exhibited central or effector memory cell characteristics, and identified the type of response through measurement of cytokines secreted into the supernatant. PBMCs from each of the 16 individuals in each group were labeled with CFSE and then cultured in the presence of whole virus. The percent of $CFSE^{dim}CD4^+$ T cells was determined by flow cytometry and used as a measure of antigen-specific cells in culture [26]. When examined as a group, there was no significant increase in the mean percent $CFSE^{dim}CD4^+$ T cells in response to H1N1, H3N2 or B viruses after vaccination with either TIV or LAIV (Figure 2). However, when examining the response of individuals, the fold increase in proliferation (ratio of proliferation on day 28 compared to day 0) was often greater for CD4⁺ T cells from volunteers in the LAIV than the TIV group (Figure 3). There was a statistically significant difference between the increased proliferation measured for H1N1-stimulated cells from LAIV than TIV recipients (p=0.02, Mann-Whitney test). Individuals were designated as responders when the increase in proliferation after vaccination (proliferative index) was greater than 2; 31% of LAIV vs 13% of TIV recipients responded to the H1N1 antigen, 44% of LAIV vs 27% of TIV recipients responded to the H3N2 antigen, and 19% of LAIV vs 13% of TIV recipients responded to the B antigen. These results suggest that there is greater antigen-specific CD4⁺ T cell stimulation after vaccination with LAIV than TIV.

CD62L and CCR7 have been used to discriminate between central memory (T_{CM}) and effector memory (T_{EM}) cells; T_{CM} are generally $CD62L^+CCR7^+$ and have high proliferative capacity but lack effector function. When T_{CM} are stimulated in vitro, CCR7 expression is lost, reflecting differentiation of these cells into effectors, with switched expression of chemokine receptors [27]. In contrast, T_{EM} cells have good effector function, and are characterized as $CD62L^-CCR7^-$ [28]. Our data showed the presence of influenza-specific T_{CM} in most volunteers prior to vaccination, and therefore CD62L and CCR7 were

Table 3: Percent of LAIV or TIV group with ≥2-fold increase in cytokine concentration post-vaccination

	Percent of vaccine group that exhibited a response[a]	
	LAIV (n=16)	TIV (n=16)
IL-2	13	13
IL-4	13	6
IL-5	13	63
IL-10	6	13
IL-12	0	19
IL-13	13	25
IFN-γ	81	19
TNF-α	44	56

[a]Cytokines were measured in supernatants of PBMCs stimulated with influenza virus or controls as described in Materials and Methods. Individuals with a ≥2-fold increase in cytokine concentration to any influenza vaccine antigen (H1N1, H3N2 or B) were considered responders.

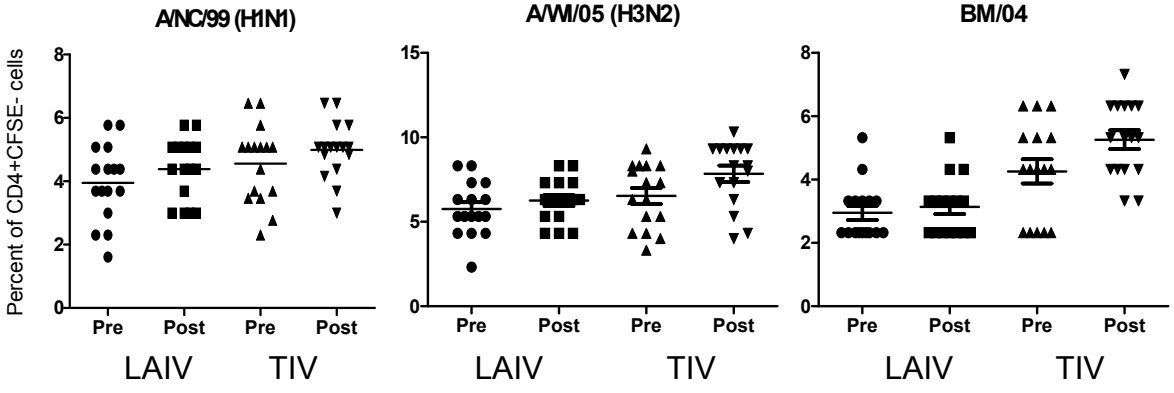

Figure 2: Percent CD4+ T cells before and after vaccination with LAIV and TIV. PBMC were collected on day 0 and 28 following vaccination with either TIV or LAIV. The cells were labeled with CFSE, and then stimulated in culture for 7 days with each virus contained in the 2006 vaccine. The percent CD4⁺ T cells that were $CFSE^{dim}$ are shown for each individual at each time point, with a group mean indicated on the graph. There was no significant difference between the group mean before and after vaccination.

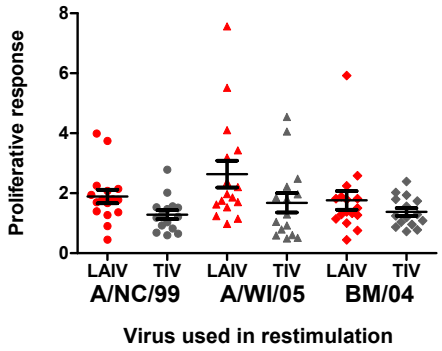

Figure 3: Fold increase in proliferative index following vaccination with LAIV and TIV. CD4+ T cells were labeled with CFSE and then cultured with whole virus A/NC/99 (H1N1), A/WI/05 (H3N2) or BM/04. The fold increase in proliferation after vaccination was calculated by determining the ratio of the day 28:day 0 proliferative index (ratio of percent $CFSE^{dim}$ cells stimulated with influenza:% $CFSE^{dim}$ cells in wells without antigen). The fold increase for each individual in the group is shown together with 95% confidence intervals for each group. The proliferative response for H1N1-stimulated cultures was significantly different between LAIV and TIV groups (p=0.02, Mann-Whitney), but not for H3N2 or B groups (p=0.07 and 0.33, respectively).

not useful as biomarkers of vaccine response (results not shown).

Comparison of cytokines secreted by antigen-specific T cells following receipt of live and inactivated vaccines

Secretion of cytokines is another measure of T cell response that may be valuable in assessment of LAIV immunogenicity. The supernatants of PBMC stimulated with each viral antigen or control were harvested on day 3 and a panel of cytokines (IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, IFNγ, and TNFα) quantified. There was no significant increase in the mean cytokine concentration in supernatants before and after vaccination for the LAIV and TIV groups (results not shown). However, individual vaccinees had significant increases in cytokine expression. An individual was identified as a responder when their cytokine concentration post-vaccination was ≥2-fold greater than the cytokine concentration prior to vaccination. Our results show that a greater proportion of individuals vaccinated with LAIV than TIV, secreted IFN-γ following restimulation with virus. In contrast, a greater proportion of individuals vaccinated with TIV had T cells secreting IL-5 (Table 3). A similar proportion of individuals in LAIV and TIV groups had increased levels of TNF-α in supernatants after whole virus restimulation. The antigen that stimulated the greatest response was A/WI/05 (H3N2); when responses to this antigen only were considered, the same pattern was observed - T cells from LAIV recipients most frequently had a Th1-type phenotype (IFN-γ expression), but T cells from TIV recipients often secreted IL-5, a cytokine characteristic of Th2-type cells.

Correlation between cellular and humoral responses

Table 4 provides an example of HI and neutralizing antibody titers against H1N1 and H3N2 viruses, together with $CD4^+$ T cell proliferation and IFN-γ and IL-5 secretion in response to these same antigens. These data illustrate how responses were defined, and show increases in neutralizing titer, even though small, that were defined as a response because they were statistically significant. As others have demonstrated [29], the greatest T cell responses were often observed in individuals with low baseline T cell levels. For the examples shown in Table 4, this is particularly evident in the LAIV responses against H1N1 and TIV responses against H3N2. One might expect that cytokine concentration would correlate with $CD4^+$ T cell proliferation; this was evident in some cases (example from Table 4, volunteer LAIV-3 had increased proliferation and increased concentration of IFN-γ after vaccination), but not others (example from Table 4, volunteer LAIV-1 had increased proliferation of cells, but no increase in IFN-γ). When we used data from all 16 individuals in each group to evaluate the correlation between proliferation, cytokine secretion, and antibody titers elicited in response to LAIV and TIV , there was no obvious correlation between any antibody measurement and cellular response, and T cell proliferation was not indicative of a cytokine response. Further statistical analyses did not show a tendency toward correlation of any cytokine and the neutralizing antibody response (Spearman's coefficient was <0.4 for all analyses), but the neutralizing antibody titer

Table 4: Examples of antibody and T cell responses to H1N1 and H3N2 antigens after vaccination with LAIV and TIV

	HI			AVINA			CD4+ T cell Proliferation			IFN-γ (pg/ml)			IL-5 (pg/ml)		
H1N1	pre	post	Response[a]	pre	post	Response[b]	pre	post	Response[c]	pre	post	Response[d]	pre	post	Response[d]
LAIV-1	5	20	R	52	199	R	1.9	7.5	4.0	82	64	0.8	59	49	0.8
LAIV-2	20	20	NR	295	473	R	2.1	4.4	2.1	106	261	2.5	48	23	0.5
LAIV-3	80	160	NR	60	140	R	5.4	9.6	1.8	78	168	2.1	10	32	3.2
TIV-1	160	160	NR	215	623	R	4.8	7.5	1.6	204	178	0.9	14	15	1.1
TIV-2	40	80	NR	341	494	R	3.4	6.8	2.0	812	650	0.8	7	9	1.3
TIV-3	80	160	NR	175	251	R	1.7	2.1	1.2	369	348	0.9	11	164	14.4
H3N2															
LAIV-1	80	80	NR	52	199	R	1.4	7.5	5.5	26	40	1.5	20	36	1.8
LAIV-2	<10	20	R	98	134	R	2.4	7.8	3.2	28	74	2.6	25	28	1.1
LAIV-3	40	160	R	55	240	R	2.1	16.1	7.6	31	237	7.7	9	8	0.9
TIV-1	20	160	R	148	672	R	1.2	5.5	4.5	19	28	1.5	8	9	1.2
TIV-2	320	320	NR	193	195	NR	4.0	16.2	4.1	1006	6927	6.9	16	33	2.0
TIV-3	20	20	NR	28	61	R	6.5	14.3	2.2	1361	1217	0.9	14	8	0.6

[a]Response is a 4-fold or greater increase in titer after vaccination. R denotes Responder; NR denotes Non-Responder.
[b]Response is a statistically significant increase in the titer determined by non-linear regression analysis
[c]Response is the proliferative index i.e. ratio of T cell proliferation after vaccination, to proliferation measured before vaccination
[d]Response is the ratio of cytokine concentration measured in supernatants before and after vaccination

and magnitude of T cell proliferation appears to be inversely related; the greatest T cell proliferation was often observed for individuals with low neutralizing antibody titer prior to vaccination, and individuals with a high neutralizing antibody titer often had lower proliferative responses. However, as can be seen for some individuals listed in Table 4, there are individuals with reasonably high neutralizing antibody titers, who also had a robust $CD4^+$ T cell proliferative response.

Evaluation of LAIV immunogenicity in adults

Our study was performed using sera and PBMC from 18-49 year olds vaccinated with either TIV or LAIV. Many of these subjects did not have a 4-fold increase in HI titer, and consideration of NI titers did not increase the percent of responding individuals. The use of AVINA, a sensitive neutralization assay developed in our laboratory [20], identified a greater number of responders in each vaccine group (Tables 2,4), with 75% of TIV recipients and 38% of LAIV recipients, responding to all 3 vaccine components. $CD4^+$ T cell responses were more robust after LAIV than TIV (Figure 3), and inclusion of individuals with H3N2-specific T cell proliferation ≥2 fold the response prior to vaccination, resulted in the identification of 100% of TIV recipients and 63% of LAIV recipients as responders. When an increase in IFN-γ secretion was used as an additional measure of response to this vaccine component, the percent of H3N2-responding individuals was further increased to 81% (Table 3). Our results therefore suggest that in an adult population, quantification of cellular response, in particular when measured by increased cytokine secretion from antigen-specific T cells, may enhance the evaluation of immunogenicity of live, attenuated influenza vaccines.

When $CD4^+$ T cell responses are measured, it is important to quantify the cytokine most likely to be induced by the vaccine – our results suggest the use of assays to evaluate IFN-γ following LAIV, but IL-5 following TIV. A recent study noted an absence of increased $CD4^+$ T cell responses in young children immunized with TIV [17]. This may reflect the assay type (intracellular cytokine immunostaining and enzyme-linked immunospot assay) and cytokine targeted (IFN-γ), rather than showing an absence of antigen-specific $CD4^+$ T cells in response to TIV. In our experience, determination of the concentration of IL-5 and IFN-γ (in addition to other cytokines) in the supernatants of antigen-activated PBMC in a multiplexed plate-based assay is a practical method to evaluate cytokine responses.

In summary, the immunogenicity of influenza vaccines in adult populations is difficult to measure due to relatively high pre-existing antibody responses, and poor increases in HI titers following LAIV. Overall, our data support measurement of neutralizing antibody titers to capture a larger number of vaccine responders. $CD4^+$ T cell proliferation and IFN-γ secretion may be additional measures of immunogenicity that can be used to identify responders to live, attenuated influenza vaccines.

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Competing Interests

The authors declare that they have no financial competing interests. The opinions or assertions contained in this report are the private views of the authors and are not to be construed as reflecting the views of the Army Medical Department, the U.S. Department of the Army, the U.S. Department of Defense and the U.S. Food and Drug Administration.

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