Live and Inactivated Influenza Vaccines Induce Similar Humoral Responses, but Only Live Vaccines Induce Diverse T-Cell Responses in Young Children

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Background. Two doses of either trivalent live attenuated or inactivated influenza vaccines (LAIV and TIV, respectively) are approved for young children (≥24 months old for LAIV and ≥6 months old for TIV) and induce protective antibody responses. However, whether combinations of LAIV and TIV are safe and equally immunogenic is unknown. Furthermore, LAIV is more protective than TIV in children for unclear reasons.

Methods. Children 6–35 months old were administered, 1 month apart, 2 doses of either TIV or LAIV, or combinations of LAIV and TIV in both prime/boost sequences. Influenza-specific antibodies were measured by hemagglutination inhibition (HAI), and T cells were studied in flow cytometric and functional assays. Highly conserved M1, M2, and NP peptides predicted to be presented by common HLA class I and II were used to stimulate interferon-γ enzyme-linked immunospot responses.

Results. All LAIV and/or TIV combinations were well tolerated and induced similar HAI responses. In contrast, only regimens containing LAIV induced influenza-specific $CD4^+$, $CD8^+$, and γδ T cells, including T cells specific for highly conserved influenza peptides.

Conclusions. Prime/boost combinations of LAIV and TIV in young children were safe and induced similar protective antibodies. Only LAIV induced $CD4^+$, $CD8^+$, and γδ T cells relevant for broadly protective heterosubtypic immunity.

Clinical Trials Registration. NCT00231907.

Vaccination is the most effective method to prevent influenza and its complications. Currently 2 types of licensed trivalent vaccines are available to prevent influenza in the United States [1]. Intramuscular trivalent inactivated vaccines (TIV) and intranasal live attenuated influenza vaccines (LAIV) are prepared from reassortment viruses expressing hemagglutinin (HA) and neuraminidase from recently circulating seasonal strains. Both TIV and LAIV are approved for young children (≥24 months old for LAIV and ≥6 months old for TIV), and both types of vaccines induce vigorous HA-specific immunoglobulin G antibodies protective against influenza infection and disease. The Advisory Committee on Immunization Practices recommends that vaccine-naive children 6 months to 8 years of age initially receive 2 doses of influenza vaccine 4 weeks apart. To date, only TIV followed by TIV or LAIV followed by LAIV have been studied. Whether prime/boost combinations of LAIV and TIV are safe and

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equally immunogenic is unknown. TIV vaccination delivers standardized amounts of viral HA (7.5 μg/strain for children 6–35 months old) [1]. LAIV contains cold-adapted influenza viruses that replicate in the nasopharynx and are shed in respiratory secretions [1]. Although both TIV and LAIV reduce influenza illness and disease complications, LAIV induces superior protection in children [2, 3]. LAIV induces influenza-specific serum antibody responses in children similar to those induced by TIV, but in addition, LAIV induces nasal secretory immunoglobulin A responses that may be important for enhanced protection [4]. Relatively little, however, has been done to compare cell-mediated immune (CMI) responses induced by TIV and LAIV in children. Enhanced CMI responses induced by LAIV could be partially responsible for the enhanced efficacy detected in children. We sought to determine whether prime/boost combinations of TIV and LAIV are as safe and immunogenic as TIV/TIV and LAIV/LAIV prime/boost regimens in children 6–35 months old. In addition, we compared both humoral and cellular immune responses induced by different prime/boost schedules of TIV and/or LAIV.

METHODS

Subjects

Healthy children 6–35 months of age were recruited from metropolitan St Louis, Missouri, in year 1 (2005–2006 influenza season), and subjects 12–35 months of age were recruited from St Louis; Cincinnati, Ohio; and Nashville, Tennessee, in year 2 (2006–2007 influenza season). The age range for recruitment was altered in year 2 after data emerged from other trials that young children (aged 6–11 months) experienced more adverse events after LAIV than after TIV [3].

Subjects were free of major medical illness, as determined by history and physical examination. A minimum weight of 8 kg was required. Subjects were excluded for hypersensitivity to vaccine components (including egg products), close contact with immunocompromised persons, and a history of immunosuppressive disease, reactive airway disease, or Guillain-Barré syndrome. All children had not received previous influenza vaccination.

Clinical Trial Design

The primary objective was to compare immunogenicity of different prime/boost regimens of TIV and LAIV. Participants were randomly assigned in equal proportions to 1 of 4 vaccine groups: group 1 (TIV/TIV), group 2 (LAIV/LAIV), group 3 (TIV/LAIV), or group 4 (LAIV/TIV).

After vaccination, subjects were observed for 30 minutes for adverse reactions. Parents and guardians were given memory aids to record adverse events for 2 weeks after vaccination. The memory aid included scoring of solicited systemic and local reactions and prompted the parent to call in case of a severe reaction.

Vaccines

LAIV (Flumist) and TIV (Fluzone) seasonal products were given 1 month apart. The vaccine strains used in 2005–2006 were A/New Caledonia/20/99(H1N1)-like, A/California/7/2004(H3N2)-like, and B/Shanghai/361/2002-like viruses. The 2006–2007 formulations contained A/New Caledonia/20/99(H1N1)-like, A/Wisconsin/67/2005(H3N2)-like (A/Wisconsin/67/2005 and A/Hiroshima/52/2005 strains), and B/Malaysia/2506/2004-like (B/Malaysia/2506/2004 and B/Ohio/1/2005 strains) viruses. The H1 antigens used for LAIV and TIV were variants of A/New Caledonia/20/99(H1N1), with 4 amino acid changes leading to significant effects on the HA-specific antibody responses induced.

Viral Cultures

Throat and nose swabs were cultured for vaccine virus and for circulating wild-type viruses if respiratory symptoms were reported. In addition, throat and nose swabs were collected from asymptomatic subjects routinely on days 0, 3–4, 5–6, 7–8, 9–10, 11–12, 13–14, and 28–32 after each dose (7 samples) in year 1 and on days 3–5 after each dose (1 sample) in year 2. Frozen samples were shipped to Saint Louis University (St Louis, MO) for culture as described elsewhere [5].

Serologic Hemagglutination Inhibition Responses

Hemagglutination inhibition (HAI) antibodies specific for all 3 HA types included in the vaccines were measured as described elsewhere [6] in plasma samples obtained on days 0 (before dose 1), 30 (before dose 2), and 60. Because of the 4 amino acid differences in the New Caledonia H1 antigens between the TIV and LAIV vaccines used in both influenza seasons, serum samples were tested against both H1 variants.

Assays of Antigen-Specific Proliferation and Interferon γ Production by T Cells

CarboxyFluorescein Succinimidyl Ester (CFSE)-labeled peripheral blood mononuclear cells (PBMCs; Molecular Probes) were expanded with peptide pools or live influenza (FluA/H3N2/California/07/04) or rested in medium for 7 days at 37°C with 5% CO_2 (adding 20 U/mL human interleukin 2 on day 4). Then cells were incubated with 50 ng/mL Phorbol 12-Myristate 13-Acetate (PMA) (Sigma), 750 ng/mL ionomycin (Sigma), and 0.7 μL/mL GolgiStop (BD) for 2 hours before CD3, CD4, CD8, and Gamma/Delta T cell Receptor (γδTCR) staining, and permeabilized with Cytofix/Cytoperm (BD), followed by intracellular interferon γ (IFN-γ) staining. Data were acquired with a FACSCalibur flow cytometer (BD) and analyzed using CELLQuest (BD) and FlowJo software (Tree Star). Absolute numbers of effector $CD4^+$, $CD8^+$, and $\gamma\delta TCR^+$ T cells (defined as both $CFSE^{low}$ and IFN-γ^+) were calculated by multiplying total viable cells times T-cell subset percentages.

IFN-γ Enzyme-Linked Immunospot Assays

Cells producing IFN-γ were identified by enzyme-linked immunospot (ELISPOT) assays using ImmunoSpot plates (Cellular Technology) and IFN-γ–specific antibodies (BD Pharmingen). The PBMCs (3×10^5 cells/well) were stimulated with peptide pools (5 μg/mL), live influenza (FluA/H3N2/California/07/04), or medium alone overnight at 37°C with 5% CO_2. Spots were identified by Analyzer and ImmunoSpot software (version 3.2; CTL).

Identification and Production of Universally Relevant Influenza-Specific T-Cell Epitopes

Influenza M1/M2 and NP protein sequences from recent seasonal H1N1, seasonal H3N2, and avian H5 and the 1918 pandemic H1N1 influenza strains were aligned, and sequences present in all strains with 100% identity were identified. Rankpep software (http://bio.dfci.harvard.edu/RANKPEP/) was used to predict peptides capable of binding common (frequency, >10% of worldwide populations) HLA class I and class II molecules. Supplementary Tables 1 and 2 (online only) present the specific peptide sequences chosen. Peptides were synthesized by JPT Peptide Technologies. Two separate pools were prepared of putative class I and class II epitopes (peptide pool 1 [PP1] and peptide pool 2 [PP2], respectively), resulting in 5 μg/mL of each peptide at assay dilution.

Measurement of T-Cell-Mediated In Vitro Inhibition of Influenza Replication

Influenza-infected monocyte and macrophage targets were prepared by plating PBMCs (150 000 cells/well) in 96-well culture plates. Nonadherent cells were gently washed away the next morning. Adherent monocytes were cultured for 6 more days in RPMI medium with glutamine, 10% human AB serum, and penicillin-streptomycin. During this same period, autologous T cells were rested, stimulated with live H3N2 influenza or peptides, or expanded with autologous blood monocyte-derived dendritic cells (DCs) prepared as described elsewhere [7] and pulsed with peptides. Interleukin 2 (10 U/mL) was added to facilitate expansion of T cells stimulated with peptides. On day 7, monocyte targets were infected with H3N2 influenza (multiplicity of infection, 0.1) for 1 hour in the presence of 100 μg/mL L-1-Tosylamide-2-Phenylethyl Chloromethyl Ketone-trypsin (Sigma) and then washed to remove extracellular virus. Rested and expanded T cells were co-cultured with influenza-infected autologous targets at Effector:Target ratios of 10:1. After 0, 4, and 24 hours, total RNA was extracted and influenza genomes were quantified by quantitative reverse-transcription polymerase chain reaction as described elsewhere [8].

Statistics

Analysis of variance (ANOVA) was used to compare geometric mean titer HAI responses across all 4 vaccination groups. Wilcoxon matched pairs tests were used to compare matched prevaccination and postvaccination responses, and Mann–Whitney *U* tests were used to compare responses between groups. Correlations were studied with Spearman rank tests. McNemar and Fisher exact tests were used to compare paired and unpaired categorical data, respectively. Analyses were completed with Statistica (Statsoft) or SPSS (at EMMES).

RESULTS

Subjects, Demographics, and Reactogenicity

Fifty-six subjects participated in the multicenter trial. Enrollment occurred during the 2005–2006 and 2006–2007 influenza seasons. Fifty-three subjects received both vaccinations and 52 completed 7 months of safety follow-up. Four withdrawals included 1 subject who developed wheezing after TIV, 1 subject who was lost to follow-up, and 2 subjects who did not meet trial requirements for reasons unrelated to vaccination. Fifty-three subjects are included in the final analysis. Demographic characteristics were similar in all 4 vaccination groups. Specifically, sex (28 male, 27 female), ethnicity (37 non-Hispanic, 18 Hispanic), race (43 white, 8 black or African American, 4 multiracial), and age (mean, 18.9 months) were well balanced. Reactogenicity is summarized in Table 1. No severe reactogenicity occurred after priming vaccinations. One elevated temperature graded as severe (>103°F; axillary) occurred after the second vaccination (TIV/TIV group) but was considered to be unrelated to vaccination. Rhinorrhea was the most common reaction, occurring in 80% of subjects after the first dose and 58% after the second dose

Table 1. Systemic and Local Reactogenicity by Vaccination Schedule

Group, dose	No. of subjects with adverse event		
	Elevated axillary temperature	Rhinorrhea	Injection site pain
TIV/TIV			
1 (n = 14)	1	10	1
2 (n = 14)	0	7	4
LAIV/LAIV			
1 (n = 13)	3	9	0
2 (n = 13)	3	9	0
TIV/LAIV			
1 (n = 13)	5	10	2
2[a] (n = 12)	1	5	0
LAIV/TIV			
1 (n = 15)	2	15	0
2[a] (n = 14)	2	10	1

NOTE. Daily monitoring was performed for days 0–13 after vaccination. An elevated axillary temperature was defined as a temperature of >99.6°F. Rhinorrhea indicates nasal drainage not associated with crying or congestion, and injection site pain indicates awareness of the symptom. LAIV, live attenuated influenza vaccine; TIV, trivalent inactivated vaccine.

[a] One subject in this group did not receive dose 2.

(similar occurrence in all vaccination groups). There were significantly fewer children with rhinorrhea after the second dose compared with the first dose ($P = .021$; Fisher exact test). There were no differences between groups regarding other solicited systemic reactogenicity. Three severe unsolicited events occurred; all were determined to be unrelated to vaccination.

LAIV Viral Shedding

Viral shedding after vaccination is summarized in Table 2 (shedding refers to recovery of influenza A and/or influenza B components of the LAIV vaccine). Viral shedding after the first dose of LAIV was detected in 17 (61%) of 28 subjects. In subjects primed with LAIV, subsequent shedding occurred in only 1 (8%) of 13 subjects following the second dose ($P = .002$; Fisher exact test compared with shedding after LAIV priming). In subjects primed with TIV, shedding occurred in 4 (31%) of 13 subjects after LAIV boosting ($P = .10$ compared with shedding after LAIV priming).

Humoral Immune Responses

Serum HAI antibody responses are summarized in Table 3. The results shown for H1N1-specific HAI responses are from assays using either LAIV or TIV A/H1N1 HA variant antigens as a target (4 amino acid differences between the 2 sequences). The amino acid differences in the TIV and LAIV H1 HA antigens resulted in differences in measured H1-specific HAI activity between the homologous and heterologous responses. H1N1-specific HAI responses were significantly higher in the TIV/TIV group than in the LAIV/LAIV group when TIV-derived H1 HA antigen was used as the HAI assay target ($P < .01$ by ANOVA after both doses 1 and 2). Conversely, H1N1-specific HAI responses were 2–3-fold higher in all 3 groups of subjects who were given LAIV at least once compared with the TIV/TIV group, when LAIV-derived H1 HA antigen was used as the HAI assay target (although these differences did not achieve statistical significance). Otherwise, there were no meaningful differences in HAI responses between the different LAIV and TIV prime/boosted groups.

Table 2. Shedding of Vaccine Virus

Group, dose	No. of subjects who tested positive/Total no. of subjects	No. of cultures that tested positive/Total no. of cultures
TIV/TIV		
1	0/14	0/16
2	0/14	0/13
LAIV/LAIV		
1	9/13	11/38
2	1/13[a]	1/40
TIV/LAIV		
1	0/8	0/21
2	4/13	4/49
LAIV/TIV		
1	8/15	12/43
2	0/7	0/15

NOTE. Nasopharyngeal swabs were collected routinely after vaccination with live attenuated influenza vaccine (LAIV) and as dictated by symptoms of illness. During year 1, the schedule was 3 times weekly for 2 weeks after LAIV vaccination; during year 2, the schedule was once on day 3–5 after LAIV vaccination.

[a] In the LAIV/LAIV group, significantly less shedding ($P = .002$; Fisher exact test) occurred after the second dose compared with the first dose.

Cellular Immune Responses

Figure 1 presents flow cytometry dot plots identifying $CD4^+$, $CD8^+$, and $\gamma\delta TCR^+$ T cells that proliferated and produced IFN-γ in rested and live influenza-stimulated PBMCs from 1 LAIV/LAIV recipient harvested before and after both doses of LAIV vaccination. The upper left quadrants of each dot plot enumerate T cells that both proliferated (became $CFSE^{low}$) and produced IFN-γ. Only small percentages of all 3 T-cell subsets proliferated and produced IFN-γ before and after vaccination after 1 week of rest prior to PMA and ionomycin stimulation (0.0%–1.4%), which demonstrates the lack of significant background responses. Influenza-specific responses were detectable in all 3 T-cell subsets before vaccination (4.3%–19.3% were $CFSE^{low}$ and IFN-γ^+ after live influenza stimulation), which is consistent with previous exposure to cross-reactive T-cell antigens. However, for all 3 T-cell subsets, marked increases in influenza-specific responses were seen after LAIV vaccination (23.8%–46.2% were $CFSE^{low}$ and IFN-γ^+ after live influenza stimulation; 2–5-fold increases compared with prevaccination influenza-stimulated responses). Figure 2 presents a composite of all $CD4^+$, $CD8^+$, and $\gamma\delta TCR^+$ T-cell responses measured with this CFSE dilution and intracellular cytokine staining assay in subjects from all 4 prime/boosted groups (10–13 subjects per group with matching prevaccination and 1-month post–dose 2 responses). The overall striking finding was that significant increases in all 3 T-cell responses were detected in the 3 prime/boosted groups of children who were given LAIV at least once. In contrast, children who received 2 doses of TIV had no detectable postvaccination increases in any of these T-cell responses.

We also performed IFN-γ ELISPOT assays with PBMCs stimulated overnight with highly conserved influenza NP and M1/M2 peptide sequences predicted to be CD4 (PP1) or CD8 (PP2) T-cell epitopes based on commonly expressed class II and class I HLA binding motifs, respectively. Figure 2 demonstrates that only children given LAIV at least once developed increased IFN-γ ELISPOT responses directed against these highly conserved influenza sequences predicted to be targets for protective T-cell recognition in the majority of individuals from highly diverse human populations.

Table 3. Comparison of Serum Hemagglutination Inhibition Antibody Responses

Variant, HAI assay target	GMT (95% CI) All subjects (n = 53)	TIV/TIV group (n = 14)	LAIV/LAIV group (n = 13)	TIV/LAIV group (n = 12)	LAIV/TIV group (n = 14)	*P*
TIV A/H1N1						
Prevaccination	4.7 (4.1–5.4)	4.6 (3.4–6.4)	5.0 (3.5–7.1)	5.3 (3.8–7.6)	4.2 (3.8–4.7)	.651
Postvaccination 1	11.3 (7.6–16.8)	21.5 (8.8–52.9)	6.0 (3.5–10.3)	25.4 (10.1–64.1)	5.1 (3.0–8.7)	.002
Postvaccination 2	32.9 (22.2–48.6)	52.5 (25.4–109)	11.3 (5.3–24.3)	42.7 (19.1–95.7)	41.0 (17.4–96.3)	.022
LAIV A/H1N1						
Prevaccination	4.6 (3.9–5.3)	4.9 (3.2–7.5)	5.0 (3.1–7.9)	4.5 (3.5–5.8)	4.0 (*)	.752
Postvaccination 1	9.5 (6.6–13.7)	8.8 (3.1–25.0)	13.5 (6.8–26.6)	8.0 (3.3–19.3)	8.8 (5.4–14.5)	.777
Postvaccination 2	22.3 (14.9–33.5)	11.3 (4.7–27.4)	28.5 (14.5–55.9)	26.9 (13.3–54.5)	30.5 (10.6–87.8)	.245
Influenza A/H3N2						
Prevaccination	9.5 (6.5–13.9)	8.0 (3.8–16.7)	10.4 (3.9–27.7)	10.7 (4.6–25.0)	9.3 (4.0–21.4)	.951
Postvaccination 1	42.9 (26.5–69.4)	43.1 (14.7–126)	30.2 (12.2–75.0)	71.8 (15.8–326)	37.1 (19.0–72.5)	.658
Postvaccination 2	88.1 (60.3–129)	86.1 (36.6–203)	47.9 (27.8–82.8)	95.9 (30.9–298)	141.3 (75.6–264)	.251
Influenza B						
Prevaccination	4.5 (3.9–5.1)	5.7 (3.4–9.5)	4.2 (3.8–4.7)	4.2 (3.7–4.8)	4.0 (*)	.228
Postvaccination 1	7.2 (5.4–9.7)	9.3 (3.7–23.5)	10.7 (5.7–20.2)	5.7 (3.6–8.8)	4.9 (4.0–5.9)	.172
Postvaccination 2	34.2 (23.6–49.6)	30.5 (13.2–70.4)	19.0 (9.9–36.6)	30.2 (11.9–76.5)	70.7 (37.1–135)	.078

NOTE. The *P* value of comparisons between treatment groups in hemagglutination inhibition (HAI) geometric mean titers (GMTs) are calculated by analysis of variance. Note that there was a 4–amino acid difference in the H1 hemagglutinin sequence between the trivalent inactive vaccine (TIV) and live attenuated influenza vaccine (LAIV) used. CI, confidence interval. * Confidence interval was not estimated as all observed values were the same.

T Cells Reactive With Universal Influenza-Specific Epitopes Can Inhibit Influenza Replication

We next studied whether T cells reactive with the universally relevant influenza epitopes designed above could recognize influenza-infected human cells and mediate inhibition of viral replication. Figure 3 presents the results from 2 separate adult volunteers using autologous DCs pulsed with the putative CD4 (PP1) or CD8 (PP2) pools to expand epitope-specific T cells that were then co-cultured with influenza-infected monocyte and macrophage targets. In both volunteers, T cells expanded with PP1-pulsed DCs resulted in 50%–90% inhibition of influenza virus replication. In addition, T cells from 1 of the volunteers expanded with PP2-pulsed DCs also inhibited influenza virus replication. These studies demonstrate that T cells reactive with influenza-specific epitopes with the potential for universal recognition of influenza

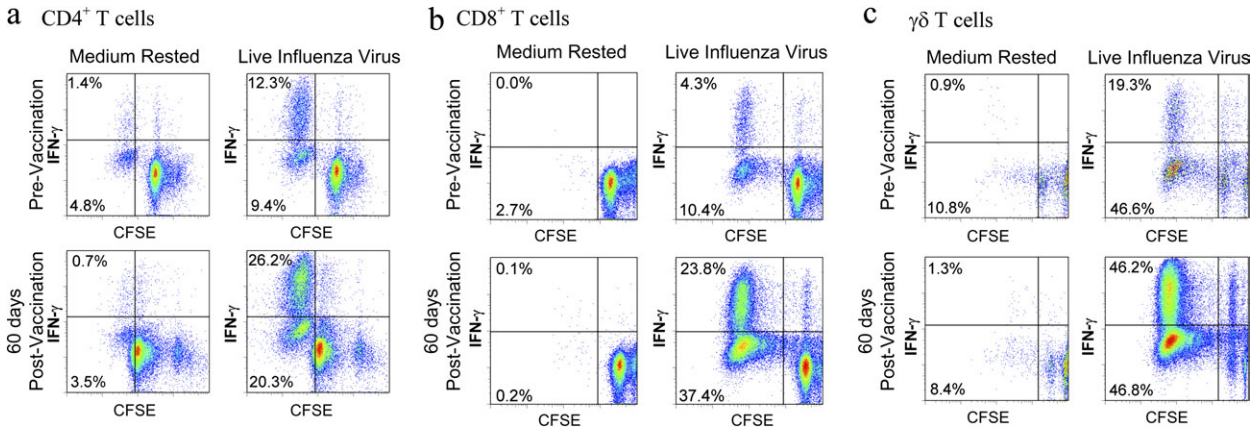

Figure 1. Induction of influenza-specific $CD4^+$, $CD8^+$, and $\gamma\delta TCR^+$ T cells capable of antigen-specific proliferative and effector cytokine responses by live attenuated influenza vaccination. Dot plots from 1 subject were gated on $CD4^+$ (*A*), $CD8^+$ (*B*), and $\gamma\delta TCR^+$ (*C*), $CD3^+$ T cells after rest (left column) or live H3N2 influenza virus stimulation (right column) for 7 days in vitro. The top and bottom rows present cellular responses before and 60 days after LAIV vaccination, respectively. The percentages given in the upper left quadrants are the percentages of T cells that both proliferated (became $CFSE^{low}$) and produced interferon γ (IFN-γ).

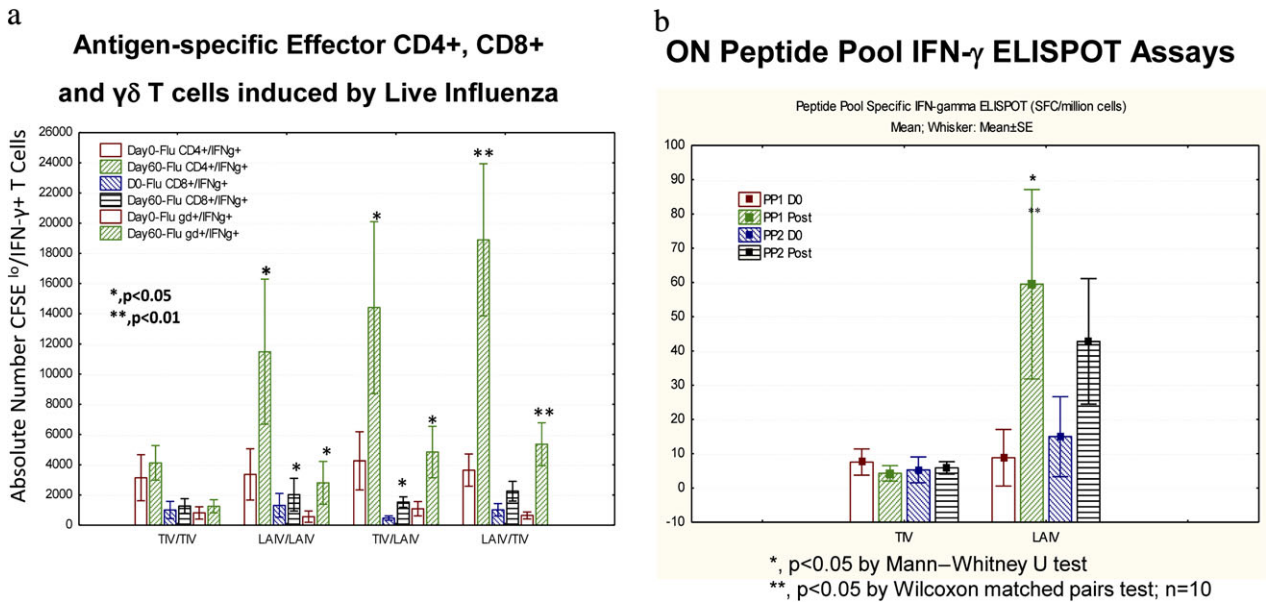

Figure 2. Live attenuated influenza vaccines (LAIV) but not trivalent inactivated vaccines (TIV) induced significantly increased postvaccination influenza-specific T-cell responses. *A,* Overall results for influenza-specific CD4 $^{+}$, CD8^{+}, and γδTCR^{+} T-cell responses induced in all prime/boosted vaccination groups. Shown are all subjects' peripheral blood mononuclear cell (PBMC) responses after live influenza virus stimulation for 7 days in vitro (matching unstimulated background responses have been subtracted). Paired responses detected in prevaccination and 1-month post-booster-vaccination PBMCs are presented (clear and shaded bars, respectively) for influenza-specific CD4^{+}, CD8^{+}, and γδTCR^{+} T cells capable of both antigen-specific proliferation and effector cytokine responses (as in Figure 1; n = 10–13 subjects per group). *$P < .05$ by Wilcoxon matched pairs test comparing prevaccination and postvaccination responses; **$P < .01$ by Wilcoxon matched pairs test comparing prevaccination and postvaccination responses. *B,* Induction of universally relevant influenza-specific T-cell responses by LAIV and not TIV vaccination. PBMCs from 10 subjects given TIV/TIV (TIV group) and 10 subjects given LAIV at least once (LAIV group) were stimulated with pools of highly conserved predicted HLA class I (peptide pool 1 [PP1]) and class II (peptide pool 2 [PP2]) peptide pools before and after vaccination and were studied by interferon γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assays (Spot Forming Cells, SFC). *$P < .05$ by Mann–Whitney U test comparing LAIV-alone responses with TIV-alone responses; **$P < .05$ by Wilcoxon matched pairs test comparing responses before and after vaccination.

strains can mediate protective responses limiting viral amplification.

Relationships Between Humoral Immunity, Cellular Immunity, and LAIV Viral Shedding

In all groups combined, CD4^{+}, CD8^{+}, and γδTCR^{+} T-cell responses were found to be highly correlated, but none of these T-cell responses were correlated with HAI responses (Table 4). Very similar results were seen when we excluded the TIV/TIV group and tested correlations between T-cell and HAI responses in only the subjects given LAIV at least once. In addition, some correlations were detected in comparisons of HAI responses directed against H1N1, H3N2, and influenza B. Furthermore, higher proportions of children who were found to shed LAIV after vaccination had 4-fold increases in both HAI antibody and T-cell responses, although these differences were not significant (data not shown).

DISCUSSION

This study examines safety and immunogenicity in various 2-dose regimens of TIV and LAIV influenza vaccines in 56 children 12–35 months of age. There were 3 reasons we undertook the study. First, LAIV appears to be more efficacious in children than inactivated vaccine; however, wheezing noted after LAIV in children <24 months of age suggests that inactivated vaccines might be a safer alternative for the initial immunization dose [2]. Therefore, children <24 months old might benefit most from TIV priming followed by LAIV boosting, but this heterologous prime/boosting strategy has not been evaluated previously in this age group. Second, patient preference and/or vaccine availability often leads to some children receiving a mixed schedule of LAIV and TIV; the current study investigated the safety and immunogenicity of such an approach. Third, we were interested in assessing the cellular immune responses induced by the 2 types of vaccines in young children to explore the possibility that cellular immune responses might be an important factor involved in the enhanced protection in children associated with LAIV vaccination.

We observed no safety issues in this small trial; however, larger trials are needed to confirm that the TIV-LAIV combination vaccine strategy in children <24 months of age is safe and effective. In addition, children receiving TIV priming followed by LAIV boosting developed humoral and cellular immune

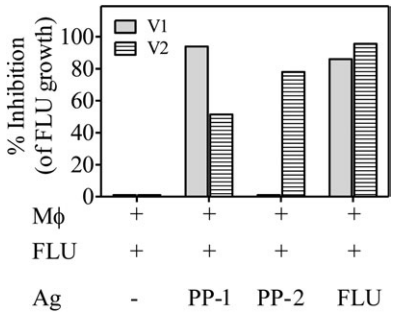

Figure 3. Inhibition of influenza viral replication by T cells reactive with highly conserved, universally relevant influenza-specific epitopes in human target cells. Peripheral blood mononuclear cells were stimulated with highly conserved, universally relevant pools of influenza peptides predicted to be $CD4^+$ and $CD8^+$ T-cell epitopes restricted by common HLA class II (PP2) and HLA class I (PP1), respectively (Ag, antigen). Live H3N2 influenza virus was used as a positive control stimulus of influenza immunity. One week later, expanded T cells were co-cultured with autologous monocyte and macrophage targets (MΦ) infected with H3N2 influenza (FLU). Twenty-four hours later, total RNA was extracted and influenza genome copies were quantified by quantitative reverse-transcription polymerase chain reaction. Shown are the percentages of influenza replication inhibition mediated by peptide-stimulated T cells compared with matching rested T cells for 2 representative adult volunteers (V1 and V2).

responses comparable to the responses detected in the other homologous or heterologous prime/boosted groups. Furthermore, LAIV priming provided significant protection against LAIV shedding when LAIV was given as the booster vaccine. Although TIV priming showed a trend of protection against LAIV shedding, these results did not achieve statistical significance. These results are consistent with those of clinical trials demonstrating that LAIV is more efficacious than TIV in children, providing further support that LAIV should be included in prime/boost regimens given to children. Large safety studies will be required to confirm this schedule in children 6–23 months old. A phase 3 comparison of TIV priming followed by LAIV boosting with homologous TIV/TIV prime/boosting would be important to perform in children <24 months of age.

The 4 vaccine regimens tested here induced similar humoral immune responses directed against H1N1, H3N2, and influenza B seasonal viral strains. These results indicate that all combinations of TIV and LAIV vaccinations should be effective against well-matched seasonal influenza strains. In addition, our results suggest that all combinations of TIV/LAIV prime/boosting vaccine schedules are likely to be safe and successful in children >6 months of age whenever there is a good match between HA expressed by the vaccines and circulating infectious viral strains. Previously, LAIV was demonstrated to induce cross-reactive antibody against a drift variant [4]—a response that likely contributes to improved protection [2, 4, 9].

In contrast, only LAIV vaccination was shown to induce influenza-specific T-cell responses relevant for cell-mediated immune protection. These results are consistent with those of previous studies indicating that LAIV induces better T-cell responses than TIV in children >5 years old and adults [10–12], but they are unique in showing that LAIV but not TIV induced $CD4^+$, $CD8^+$, and $\gamma\delta TCR^+$ T-cell responses capable of both proliferation and effector function in children 6–35 months of age. LAIV induced vigorous cellular immune responses regardless of whether it was given before or after TIV; however, subjects who received only TIV did not develop detectable memory T-cell responses in peripheral blood. These unique effects of LAIV are likely to be important for the high efficacy of a single dose of LAIV as shown in clinical studies [6, 13, 14]. In addition, these enhanced cellular immune responses may help to explain why LAIV was shown in a head-to-head trial in children 6–59 months of age to be significantly more protective than TIV against culture-confirmed influenza; LAIV was 55% more protective for both antigenically well-matched and drifted viruses [2]. Studies

Table 4. Spearman Rank Order Correlations Between T-Cell and Hemagglutination Inhibition Responses

	Correlation on day 60					
	T-cell response			HAI response		
Variable on day 60	$CD4^+$IFN-γ^+	$CD8^+$IFN-γ^+	$\gamma\delta^+$IFN-γ^+	Influenza A/H1N1	Influenza A/H3N2	Influenza B
T-cell response						
$CD4^+$IFN-γ^+	1.000	0.716[a]	0.772[a]	−0.239	−0.077	−0.166
$CD8^+$IFN-γ^+	…	1.000	0.549[a]	0.009	0.043	−0.032
$\gamma\delta^+$IFN-γ^+	…	…	1.000	−0.229	−0.009	−0.011
HAI response						
Influenza A/H1N1	…	…	…	1.000	0.497[a]	0.492[a]
Influenza A/H3N2	…	…	…	…	1.000	0.415[a]
Influenza B	…	…	…	…	…	1.000

NOTE. Shown are the *r* values for Spearman rank tests (all groups and all cases, no exclusions). HAI, hemagglutination inhibition; IFN-γ, interferon γ.

[a] Statistically significant correlations ($P < .05$).

in elderly individuals also have shown that LAIV induces better heterosubtypic immunity than TIV, in terms of both humoral and cellular immune responses [15]. Furthermore, many animal studies have indicated that influenza-specific T cells can provide broadly heterosubtypic protective immunity [16–21]. Current data also indicate that T-cell responses can provide heterosubtypic influenza-specific protective immunity [22–25]. We now demonstrate that LAIV induces conventional αβ T-cell responses that are reactive with highly conserved influenza-specific peptide pools relevant for universal influenza heterosubtypic protective immunity. Furthermore, these T cells that are reactive with highly conserved influenza-specific peptides can inhibit viral replication in human cells (Figure 3). All of these results indicate that LAIV may be able to induce better protection against antigenically drifted influenza strains and even major pandemic strains.

Conventional $CD4^+$ and $CD8^+$ αβ T cells can provide helper effects for immunity and direct inhibitory effects on viral replication. $CD4^+$ and $CD8^+$ αβ T cells capable of recognizing highly conserved influenza epitopes are relevant for broadly heterosubtypic protective immunity. If T cells are specific for conserved influenza peptides presented by common HLA alleles, they could be broadly protective in highly diverse human populations. We have recently shown that γδ T cells induced by BCG vaccination [26] and/or live viral vectors (vaccinia [27] and canarypox [28]) develop memory responses [26], undergo antigen-specific focusing [29], and can potently inhibit intracellular pathogen replication [26, 29, 30]. One report of γδ T cells having inhibitory effects on influenza viral replication in human monocytes has been published, although the antigen specificity of these inhibitory effects was unclear [31]. The induction of γδ T cells by LAIV also may provide additional protective effects against influenza.

Influenza-specific T-cell responses induced by LAIV were correlated with each other but not with humoral immune responses (Table 4). Both influenza-specific humoral and cellular immune responses were induced in subjects with detectable LAIV shedding after the priming vaccination. It is expected that LAIV replication is important for immunogenicity and also the induction of protective immunity. In addition, our combined findings that influenza-specific T-cell and B-cell responses did not correlate, but both could be induced by prolonged LAIV shedding and are likely to be important for protective immunity, may explain differences in vaccinated persons with similar protective resistance against influenza but variable influenza-specific HAI antibody titers.

In summary, our results indicate that combinations of TIV and/or LAIV are safe and effective in inducing protective antibody responses against matching seasonal strains of influenza. Despite these similarities, striking differences in cellular immune responses were seen. Only LAIV induced T-cell responses that are potentially important for protection against both matching seasonal and heterosubtypically diverse strains of influenza. TIV priming followed by LAIV boosting may be the best prime/boost regimen for use in children <24 months old. Larger clinical trials are needed to confirm the safety and efficacy of this approach. Furthermore, LAIV vaccination may induce more broadly heterosubtypic influenza-specific protective immune responses.

Supplementary Data

Supplementary Data are available at *The Journal of Infectious Diseases* online.

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