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Early Induction of Cross-Reactive CD8$^+$ T-Cell Responses in Tonsils After Live-Attenuated Influenza Vaccination in Children

K. G.-I. Mohn,[1,2,4] K. A. Brokstad,[3] S. Islam,[1,2] F. Oftung,[8] C. Tøndel,[5] H. J. Aarstad,[6] and R. J. Cox[1,2,7]

[1]Influenza Centre, University of Bergen, Bergen, Norway, [2]K. G. Jebsen Center for Influenza Vaccines, University of Bergen, Bergen, Norway, [3]Broegelmann Research Laboratory, Department of Clinical Science, University of Bergen, Bergen, Norway, [4]Emergency Care Clinic, Haukeland University Hospital, Bergen, Norway, [5]Department of Pediatrics, Haukeland University Hospital, Bergen, Norway, [6]Department of Otorhinolaryngology/Head and Neck Surgery, Haukeland University Hospital, Bergen, Norway, [7]Department of Research and Development, Haukeland University Hospital, Bergen, Norway, [8]Department of Infectious Disease Immunology, Norwegian Institute of Public Health, Oslo, Norway

Background. Live-attenuated influenza vaccine (LAIV) was licensed for prophylaxis of children 2–17 years old in Europe in 2012 and is administered as a nasal spray. Live-attenuated influenza vaccine induces both mucosal and systemic antibodies and systemic T-cell responses. Tonsils are the lymph nodes serving the upper respiratory tract, acting as both induction and effector site for mucosal immunity.

Methods. Here, we have studied the early tonsillar T-cell responses induced in children after LAIV. Thirty-nine children were immunized with trivalent LAIV (containing A/H1N1, A/H3N2, and B viruses) at days 3, 7, and 14 before tonsillectomy. Nonvaccinated controls were included for comparison. Tonsils and peripheral blood (pre- and postvaccination) were collected to study T-cell responses.

Results. Tonsillar and systemic T-cell responses differed between influenza strains, and both were found against H3N2 and B viruses, whereas only systemic responses were observed against A/H1N1. A significant increase in cross-reactive tonsillar CD8$^+$ T cells recognizing conserved epitopes from a broad range of seasonal and pandemic viruses occurred at day 14. Tonsillar T cells showed significant cytokine responses (Th1, Th2, and granulocyte-macrophage colony-stimulating factor).

Conclusions. Our findings support the use of LAIV in children to elicit broadly cross-reactive T cells, which are not induced by traditional inactivated influenza vaccines and may provide protection to novel virus strains.

Keywords. children; IFN-γ; LAIV; T cells; tonsils.

Young children carry a considerable burden of influenza disease, with the World Health Organization estimating that 10%–30% of children are infected each year. Each year, influenza has been reported to cause 20.5 million cases of severe lower respiratory tract infections in children <6 years old [1]. Inactivated influenza vaccines (IIV) are approved for use in children 6 months old or older. Inactivated influenza vaccines mainly confer protective immunity by inducing strain-specific antibodies. Due to the continuous antigenic drift and occasional shift, there is an urgent need for vaccines capable of inducing broader protection. Historical evidence showed that in the absence of influenza-specific antibodies, influenza-specific CD8$^+$ and CD4$^+$ T cells play an important role in recovery from influenza infection [2, 3]. The importance of naturally occurring T-cell immunity in protection against seasonal and pandemic influenza was recently demonstrated in a large population-based study [4]. Furthermore, CD8$^+$ T cells from the lungs of patients with influenza A or B were found to cross-react to influenza A, B, and C viruses [5], an important finding for the design of future universal influenza vaccines.

Moreover, CD8$^+$ cells were associated with less severe pandemic infection in 2009 and lower viral shedding, as well as increased survival after H7N9 avian influenza infection in China [6, 7]. Furthermore, numerous studies have demonstrated that individuals have T-cell subsets with cross-reactivity to influenza A strains to which they have not been previously exposed [8–12]. Hence, cross-reactive CD8$^+$ T cells recognizing conserved internal influenza epitopes are an interesting research focus in the development of universal vaccines.

In 2012, a live-attenuated influenza vaccine (LAIV) was licensed for children 2–17 years old in Europe. Live-attenuated influenza vaccine is administered as a nasal spray and more closely resembles a natural infection inducing long-lasting systemic humoral and cellular immune responses [13, 14]. In meta-analysis studies, the LAIV has high efficacy in children <6 years old when the vaccine strains matched the epidemic strains [3,

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Correspondence: Kristin G.-I. Mohn, The Influenza Centre, Department of Clinical Sciences, University of Bergen, The Laboratory Building 5th Floor, Haukeland University Hospital, N-5021 Bergen, Norway (kristin.mohn@uib.no).

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15, 16]. However, studies have shown protection after LAIV also in seasons with strain mismatch, indicating a broader protective effect that we are currently not able to quantitate. At this time, there are no well established immunological correlates of protection (COP) after LAIV, although induction of T cells has been highlighted. In a large field study, the majority of subjects with high frequencies of interferon (IFN)-γ-secreting cells (≥100 spot-forming cells per million lymphocytes) were protected from influenza infection, although the specific phenotype of these IFN-γ-secreting cells was not defined [3]. Furthermore, significant increases in IFN-γ^+ $CD4^+$ and $CD8^+$ T cells were observed after LAIV immunization, but not after IIV in children aged 5–9 years old. In adults, no increase in T or natural killer cells after IIV or LAIV vaccination was found [17]. Moreover, low baseline levels of T cells correlate with higher responses after vaccination [18]. When comparing the effect of different prime-boost strategies for LAIV and IIV, only regimes including LAIV induced $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells specific for highly conserved influenza epitopes [19].

Intranasal administration of vaccines has several advantages over parenteral administration, with the potential of stimulating both local and systemic immune responses, as well as being easy to administer and needle-free. Human nasopharynx-associated lymphoid tissue, which consists of adenoids and tonsils, plays an important role in immune defense of the upper respiratory tract, both as an inductor and effector site of adaptive humoral and cellular immunity [20].

We have shown proof of concept that the LAIV induced systemic T cells cross-reactive to drifted strains, providing potential clinical protection [21]. An unanswered question is whether LAIV induces T cells in the local draining lymph nodes, which could provide broad protection. Our current study is a continuation of earlier work, in the same cohort, where we showed induction of tonsillar B cells and local immunoglobulin A after LAIV [22]. In this study, we focused on the early T-cell responses in the local tonsillar tissue, to investigate whether LAIV induced cross-reactive $CD8^+$ T cells in the tonsils. To study tonsillar T cells, we vaccinated children with LAIV at specified time intervals before elective tonsillectomy, which allowed us to elucidate local, tonsillar-specific, and systemic T-cell responses. Increased understanding of these cross-protective cellular immune responses induced by LAIV may aid design of a future universal vaccine.

MATERIAL AND METHODS

Study Design

Fifty-five healthy children (3–17 years old) were recruited from the Otorhinolaryngology outpatient clinic at Haukeland University Hospital, Norway. Thirty-nine children (20 boys and 19 girls, median age 4 years) were immunized with the trivalent LAIV (Fluenz; AstraZeneca) in 2012–2013 at 3, 7, or 14 days before elective tonsillectomy. Controls consisted of age-matched unvaccinated children scheduled for tonsillectomy, providing a background comparison for postvaccination tonsillar responses (Figure 1). The study was approved by the Ethical Committee of Western-Norway and the Norwegian Medicines Agency (www.clinicaltrials.gov: NCT01866540). Demographics and inclusion and exclusion criteria for this trial have been published, and this work is a continuation of earlier findings to decipher the immune profiling after LAIV [13].

Live-Attenuated Influenza Vaccine

Trivalent LAIV contained 10^7 fluorescent focus units of A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), and B/Wisconsin/1/2010. Live-attenuated influenza vaccine was administered intranasally as a 0.1-mL spray dose per nostril. Twenty-nine children <9 years old received 2 doses of vaccine 28 days apart as recommended by the manufacturer.

Sample Collection

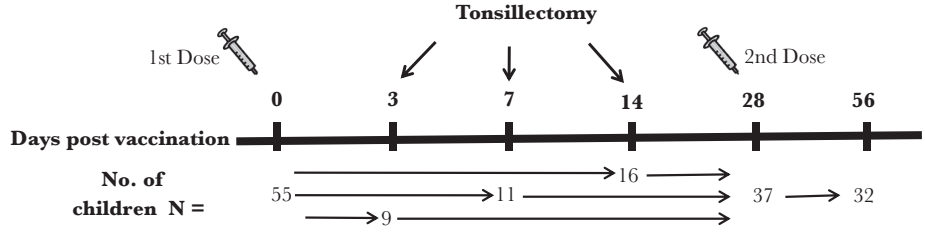
Blood samples were collected pre- and postvaccination (days 0 [at tonsillectomy], 28, and 56). Cell preparation tubes (BD Biosciences) were used to separate peripheral blood mononuclear cells (PBMCs) and plasma. Peripheral blood mononuclear cells were used fresh in the T-cell assays, whereas plasma samples were aliquoted and stored at −80°C before use in the hemagglutination inhibition (HI) assay. Tonsils were collected during the operation and kept in saline, and tonsillar mononuclear cells (TMCs) were isolated by Ficoll gradient centrifugation and used directly in the T-cell assays [23].

Antigens and Peptides

Split virus antigens from the vaccine strains A/California/7/09(H1N1), A/Victoria/361/2011(H3N2) and B/Wisconsin/1/2010, were provided by GlaxoSmithKline, Belgium. By using the Immunome Epitope Data Base, a panel of cross-reactive $CD4^+$ (33 peptides) and $CD8^+$ (31 peptides) T-cell epitopes were selected from influenza isolates spanning from 1934 to 2009, according to sequence conservancy, human leukocyte antigen (HLA) supertype coverage, and prevalence [24]. Only the peptides with the highest conservancy score were selected among the CD4 or CD8 T-cell epitopes to detect cross-protective responses using 2 and 7 peptides from the internal proteins, respectively (Supplementary Table 1). These peptide epitopes have been empirically shown to differentiate between CD4 and CD8 T-cell responses. The peptides were chemically synthetized by Fmoc chemistry (Mimotopes, Clayton, Australia) and dissolved in 100% dimethyl sulfoxide at 20 mg/mL.

Hemagglutination Inhibition Assay

The influenza strain-specific HI antibody was measured pre- and postvaccination. Plasma samples were treated with receptor destroying enzyme ([RDE] Seiken, Japan). Duplicate samples from each subject (starting dilution of 1:10) were tested at



Vaccinees	39[#]	29[1)]
Male/Female	20/19	
Previously vaccinated	21	
Median age (range)	4 (3,5–17yrs) 3,5 (3–14) 4 (3–15) 4,5(3 –17)	
Controls	16[*]	
Male/Female	8/8	
Previously vaccinated	6	
Median age (range)	4,5 (3–17yrs)	

• Non-vaccinated controls. # One vaccinated child provided samples on day of tonsillectomy, but no sample day 0

1) Only children under the age of 10 years old required two doses of LAIV. Two children <10 did not receive a second dose, one child was sick on the day of second vaccination and another child withdrew from the study due to post operative discomfort.

2) The patients had both their tonsils removed in one operation, and therefore tonsils were only sampled at a single time-point. Non-vaccinated controls were used as pre-vaccination (day 0) comparator for tonsillar samples. Tonsils were collected from vaccinated children at 3 or 7 or 14 days post vaccination. Serum samples were collected at multiple time-points from each vaccinated subject and at only a single time point at tonsillectomy from the non-vaccinated controls.

Figure 1. Study design. Healthy, young children were recruited from the Department of Otorhinolaryngology scheduled for elective tonsillectomy and vaccinated intranasally with a live-attenuated influenza vaccine (LAIV) at 3, 7, or 14 days before tonsillectomy. Tonsils were extracted in total and collected from the operation theater from vaccinated children (n = 39) and a group of matched nonvaccinated controls (n = 16). Tonsil mononuclear cells were separated from the tonsils immediately after operation and used in the T-cell assays. Blood samples were taken before vaccination, at the time of tonsillectomy, and up to 56 days postvaccination. Peripheral blood mononuclear cells were separated and used in the T-cell assays, and plasma was stored for use in the hemagglutination inhibition assay. The number of subjects providing samples at each time point is shown.

the same time, using 8 hemagglutinating units of the homologous H1N1 and H3N2 vaccine strains or ether-treated B virus (Influenza Reagent Resources) and 0.7% turkey red blood cells [25]. An HI titer of 40 has been shown to be protective in adults.

Interferon-γ Enzyme-Linked Immunospot Assay

Secretion of IFN-γ from TMCs and PBMCs were detected as described earlier [13] by using an IFN-γ ELISpot kit (Mabtech AB, Sweden). In brief, 4×10^5 lymphocytes/well were added in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum with negative control (medium alone), split virus antigen, or peptides at a concentration of 2 μg/mL. Plates were incubated overnight (37°C, 5% CO_2) and developed the next day according to the manufacturer's instructions. Spots were counted using an Immunoscan reader and associated software (CTL Europe). The negative control (medium alone) value was subtracted from the influenza-specific (A/H1N1, A/H3N2, B, or peptide panel) responses.

Multiplex Cytokine Assay

Tonsillar mononuclear cells and PBMCs (1×10^6 cells/well) were incubated in lymphocyte medium for 72 hours in the presence of a mixture of 2.5 μg/mL of 3 split influenza antigens (A/H1N1, A/H3N2, and B), as previously described [26]. The cytokines present in the supernatants were quantified using a 10-Plex kit (LHC0001M; Thermo Fisher Scientific) using a Luminex 100 machine (Luminex Corporation) and StarStation v.3.0 Software (Applied Cytometry, UK).

Statistical Analysis

Differences between pre- and postvaccination responses were analyzed by non-parametric Kruskal-Wallis multiple comparisons test or the Mann-Whitney test using GraphPad Prism version 6 for Mac OS X. The correlation analysis was performed by non-parametric Spearman correlation. $P < .05$ was considered significant.

RESULTS

Early Hemagglutination Inhibition Antibody Responses After Live-Attenuated Influenza Vaccine

The influenza-specific HI responses were measured after LAIV. The majority of subjects (59%) had prevaccination HI titers ≥40 to H1N1, and no increase in antibodies was observed after vaccination, except for 1 subject (Figure 2). H3N2-specific HI titers ≥40 were observed in 49% of children prevaccination, and titers increased from day 14 with all subjects having titers ≥40 at day 56. Most children (89%) had no prevaccination HI antibodies to influenza B, but antibodies increased at day 14. By 56 days postvaccination, 84% of children had titers ≥40. The nonvaccinated controls had similar antibody titers to the prevaccination titers of the vaccinees, supporting their use as relevant controls for analysis of tonsillar T cells.

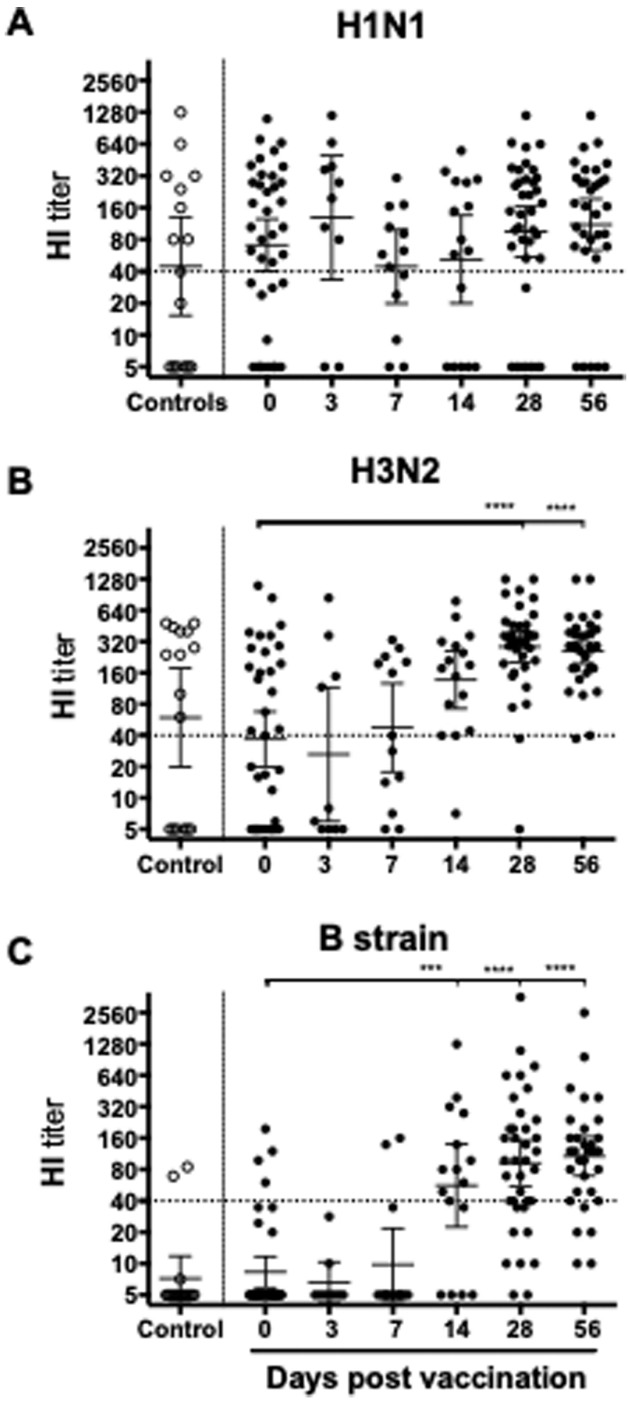


Figure 2. Serum hemagglutination inhibition (HI) antibody response after live-attenuated influenza vaccine (LAIV). Plasma was collected pre- and postvaccination including at the time of tonsillectomy from children vaccinated with LAIV. The data show the influenza A H1N1 (A), influenza A H3N2 (B), and (C) B-strain specific HI responses of each individual subject. Influenza strain-specific HI antibody was measured by HI assay, prevaccination (day 0), the day of tonsillectomy (day 3, 7, or 14), and days 28 and 56 postvaccination. Control refers to the nonvaccinated group, which had similar HI titers as the day 0 vaccinees supporting their use as controls for the tonsillar results. The horizontal lines represent the geometric mean titers ± 95% confidence interval. The dotted line represents an HI titer of 40 regarded as protective antibody titers.

Interferon-γ T-Cell Responses in Tonsils and Blood

Antigen-specific IFN-γ responses were measured in TMCs and PBMCs from LAIV-vaccinated and control subjects after stimulation with either split antigens (Figure 3) or peptides representing conserved $CD4^+$ and $CD8^+$ T-cell epitopes (Figure 4). Low levels of H1N1-specific IFN-γ-secreting TMCs were detected in nonvaccinated controls, and no increase in IFN-γ-secreting TMC response was detected after vaccination. These findings were confirmed by using $CD4^+$ and $CD8^+$ H1N1-specific peptides (Supplementary Figure 1). In contrast, the H1N1-specific IFN-γ response in PBMCs was significantly enhanced from day 0 and 3 to 56 days postvaccination (means = 58–167 and 9–167 spot-forming units [SFU]/1 × 10^6 cells, respectively), with a peak reached at day 14 (mean 200 SFU/1 × 10^6 cells). The H3N2-specific IFN-γ response of TMC was higher 14 days postvaccination compared with controls (day 14 mean = 181 and control = 80 IFN-γ SFU/1 × 10^6 cells, respectively), although not statistically significant. Vaccination did not significantly enhance the H3N2-specific IFN-γ response in PBMCs. In contrast, both the tonsillar and the systemic PBMC B-strain-specific IFN-γ responses were significantly higher 14 days postvaccination compared with the nonvaccinated subjects (tonsillar mean = 134 and systemic mean 325 versus nonvaccinated tonsillar mean = 18 and systemic mean = 58 IFN-γ SFU/1 × 10^6 cells, respectively).

Interferon-γ $CD4^+$ and $CD8^+$ T-Cell Responses in Tonsils

To further determine the $CD4^+$ and $CD8^+$ T cells with cross-reactive potential, elicited by LAIV, we used peptide epitopes from internal influenza antigens, which are highly conserved among viral strains over several decades. After stimulation with the conserved influenza-specific $CD4^+$ (Figure 4A) or $CD8^+$ (Figure 4B) peptides, a significant increase in $CD8^+$ T-cell responses between the nonvaccinated controls and the LAIV immunized children at 14 days postvaccination was seen, indicating cross-reactive responses (means = 12 and 45 IFN-γ SFU/1 × 10^6 cells, respectively). Likewise, a nonsignificant trend towards increased $CD4^+$ T-cell responses was observed (means = 5 and 28 IFN-γ SFU/1 × 10^6 cells, at days 0 and 14, respectively). Although the TMC numbers are low, they represent a substantial number of T cells, because the total number of lymphocytes in the tonsils is large (10^9). Some subjects remained nonresponders to the peptides, indicating that the donor is antigenically naive or lacks peptide presentation due to HLA mismatch.

Cytokine Responses in Tonsils and Blood After Live-Attenuated Influenza Vaccine

Tonsillar mononuclear cells and PBMCs were stimulated with a mixture of the trivalent split vaccine antigens, and the cytokine

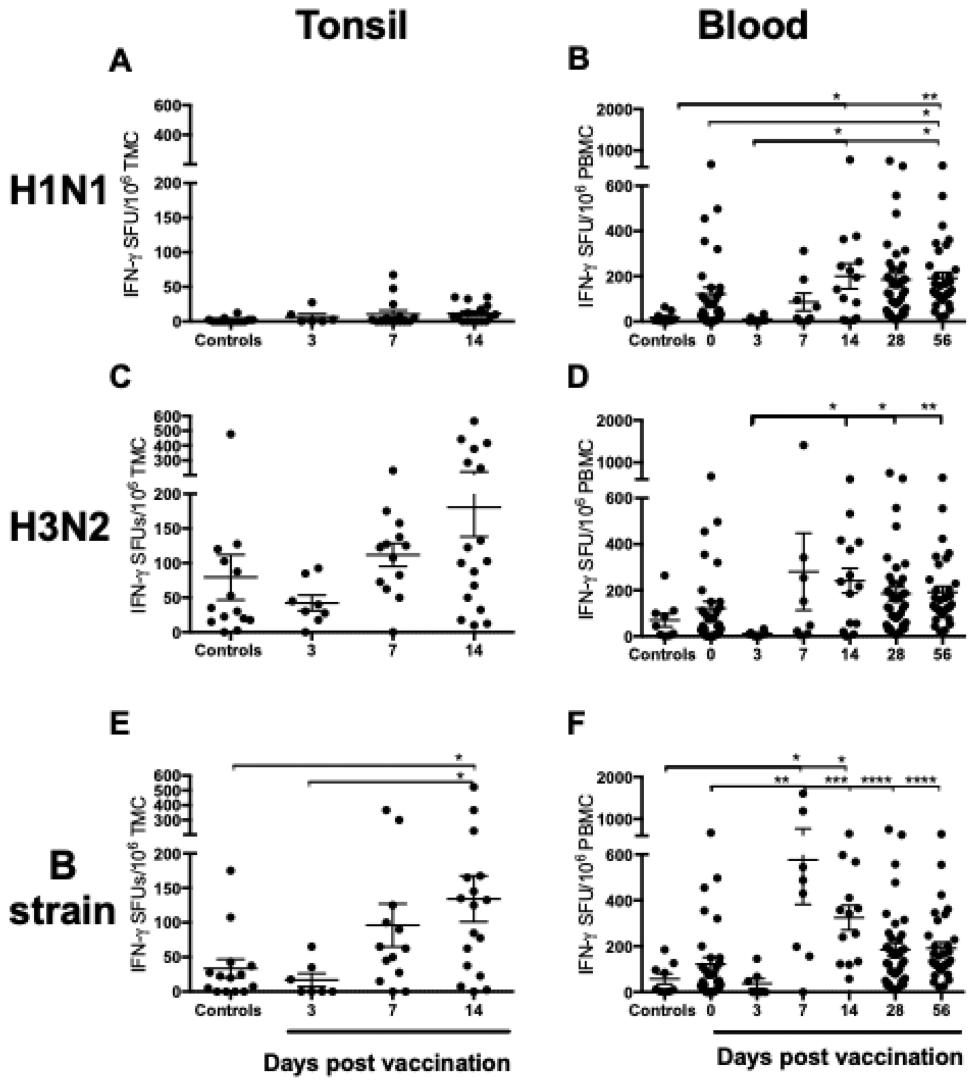


Figure 3. Strain-specific T-cell responses in tonsils and peripheral blood mononuclear cells (PBMCs) after live-attenuated influenza vaccination (LAIV). The influenza H1N1, H3N2, and B strain-specific interferon (IFN)-γ responses in tonsillar mononuclear cells ([TMC] A, C, and E) and PBMCs (B, D, and F) were determined by IFN-γ enzyme-linked immunospot in nonvaccinated controls and subjects vaccinated with the LAIV. Each symbol represents the influenza-specific IFN-γ response (spot-forming units [SFU] per 1×10^6 cells) after stimulation with split virus antigens. The horizontal bars represent the mean IFN-γ response for each time point ± standard error of the mean. Statistical significance was determined by the non-parametric Kruskal-Wallis multiple comparisons test (*, $P < .05$; **, $P < .005$).

responses detected after LAIV were grouped as Th1 (interleukin [IL]-2, IFN-γ, and tumor necrosis factor [TNF]-α), Th2 (IL-4, IL-5, IL-10, and IL-13), Th17 (IL-17), or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Figure 5). Low levels of Th1-cytokines (IL-2, IFN-γ, and TNF-α) were observed in the unvaccinated controls, whereas a significant increase of IL-2 and IFN-γ levels was detected at day 14. The levels of Th2 cytokines (IL-4 and IL-13) as well as GM-CSF increased over time postvaccination, with day 14 levels being significantly higher than the controls. However, no significant increases were observed for IL-10, IL-5, or IL-17 during the same observation period. No significant increases in cytokine responses in PBMCs (Th1, Th2, Th17, or GM-CSF) were observed postvaccination compared with prevaccination (data not shown).

DISCUSSION

T cells contribute to the protection against severe influenza illness and in recovery from infection [2]. Influenza-specific cytotoxic $CD8^+$ T cells have been linked to reduced viral shedding and increased survival after H7N9 avian influenza infection [6, 7]. Furthermore, pre-existing T-cell immunity has been found to protect against confirmed influenza disease in the community [4]. T cells often provide broad cross-reactive immune responses due to preferential recognition of epitopes from conserved internal influenza antigens [8–12]. Our study is the first to describe the induction of influenza cross-reactive CD8 T cells in the tonsils of healthy children after LAIV immunization. We used peptides representing conserved CD8

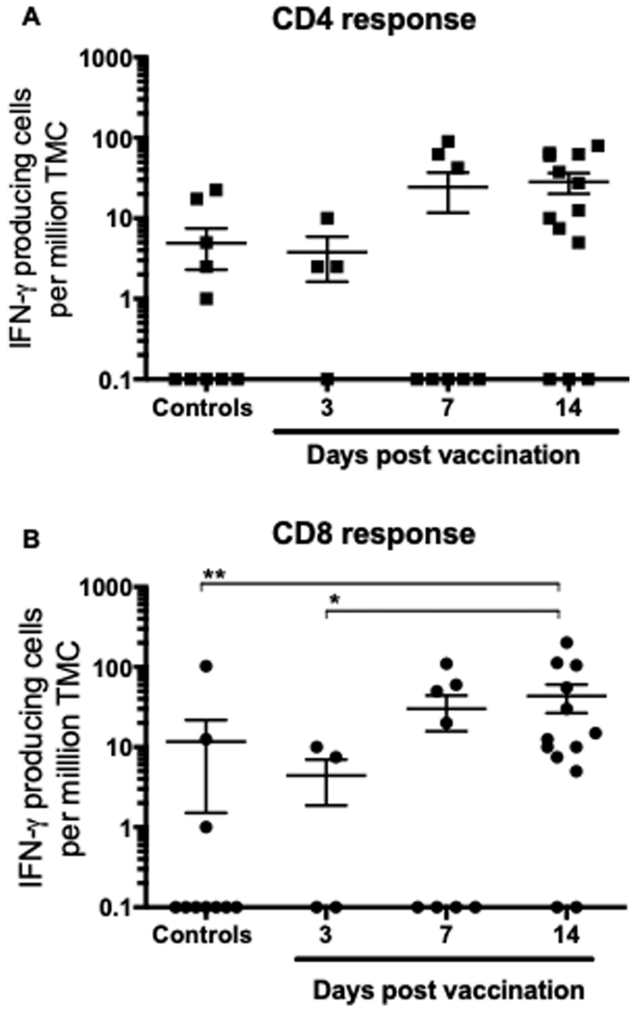


Figure 4. Cross-reactive CD4$^+$ and CD8$^+$ T-cell responses after live-attenuated influenza vaccination (LAIV) vaccination. The T-cell immune response was evaluated by measuring the number of influenza-specific interferon (IFN)-γ-secreting T cells (spot-forming units [SFU]) after LAIV, using the enzyme-linked immunospot assay. Tonsillar mononuclear cells (TMC) isolated from tonsils were tested for responses against panels of peptides representing conserved T-cell epitopes (A and B). Responses to CD4 T-cell epitopes (major histocompatibility complex [MHC] class II restriction) are shown to the left, and responses to CD8 epitopes (MHC class I restriction) are shown to the right of the figure. Each symbol represents the number of influenza-specific SFU per million TMC for each child with the mean and stand error of the mean shown. Statistical differences between vaccinated and nonvaccinated subjects were determined by the non-parametric Kruskal-Wallis (*, $P < .05$; **, $P < .005$).

T-cell epitopes, and we found that tonsillar CD8$^+$ T cells with cross-protective potential were elicited as early as 7–14 days postvaccination. This early mucosal T-cell response, close to the anatomical site of vaccine application, may provide protection at a population level, lessening the societal burden from drifted or shifted strains.

Interferon-γ has powerful antiviral activity, and increased levels may help prevent severe influenza illness [27, 28]. Elevated numbers of IFN-γ-producing T cells were detected in the blood after LAIV vaccination, lasting up to 1 year as previously described [13, 17, 19, 29, 30]. In this study, we have assessed the local influenza-specific IFN-γ response by enzyme-linked immunospot (ELISPOT) using split virus antigens, which mainly detect CD4$^+$ T-cell responses. Differences in the IFN-γ T-cell responses were observed between the 3 vaccine strains. Responses to the B strain were significantly elevated in both tonsils and blood, whereas the increase detected against H3N2 occurred in the tonsils but did not reach significance. In contrast, the H1N1-specific response increased only in the blood. The study was conducted 3 years postpandemic, and the immune response appears to be influenced by the higher prevaccination HI titers towards H1N1, which could have reduced viral replication and hence the local tonsillar immune response. Lower prevaccination titers were found towards the influenza H3N2 and B strains, with the strongest tonsillar response towards the B strain, suggesting less exposure to influenza B and efficient replication of the B strain. Differences in LAIV effectiveness data between the United States and Europe have been found, which may be due to regional differences in vaccination strategies and infection pressure [31–33].

We observed a mixed cytokine response in blood and tonsils of both Th1 and Th2 signatures, indicating the vaccine stimulates a broad immune response, with a wide range of immune competent cells involved. More important, we did not see significant increases in influenza-specific cytokine levels in the blood up to day 14 postvaccination, corresponding to the findings of low reactogenicity after vaccination. However, we have previously observed a significantly elevated systemic multifunctional CD4$^+$ response (IFN-γ, IL-2, and TNF-α) after 2 doses of LAIV in children [13]. This suggests that cytokine responses can be detected earlier in tonsils compared with blood. In agreement with this, we have previously found an early increase in tonsillar B cells and Tfh cells after LAIV [22, 34].

A postpandemic study found that the level of pre-existing cellular immunity was inversely correlated to HI antibodies [35]. This raised concerns that vaccination of children with IIV may not induce cross-reactive T-cell immunity, because IIVs primarily induce antibodies and not cellular responses [36]. However, this concern does not apply to LAIV, supported by our findings in the pediatric population that LAIV induced both broad cellular and humoral immune responses. Studies have also shown that animals challenged with heterosubtypic influenza strains were protected after LAIV [37–40]. More important, human studies have found that LAIV provided protection in children against a drifted H3N2 variant virus, naturally occurring during the studies and not contained in the vaccine [19, 41]. Furthermore, we have previously shown proof of concept that LAIV boosts cross-reactive, protection associated systemic CD8$^+$ T-cell responses after LAIV, which could provide broad immunity to drifted and shifted influenza strains [21]. However, it is not known whether these T cells provide protection at the site of infection in the upper respiratory tract.

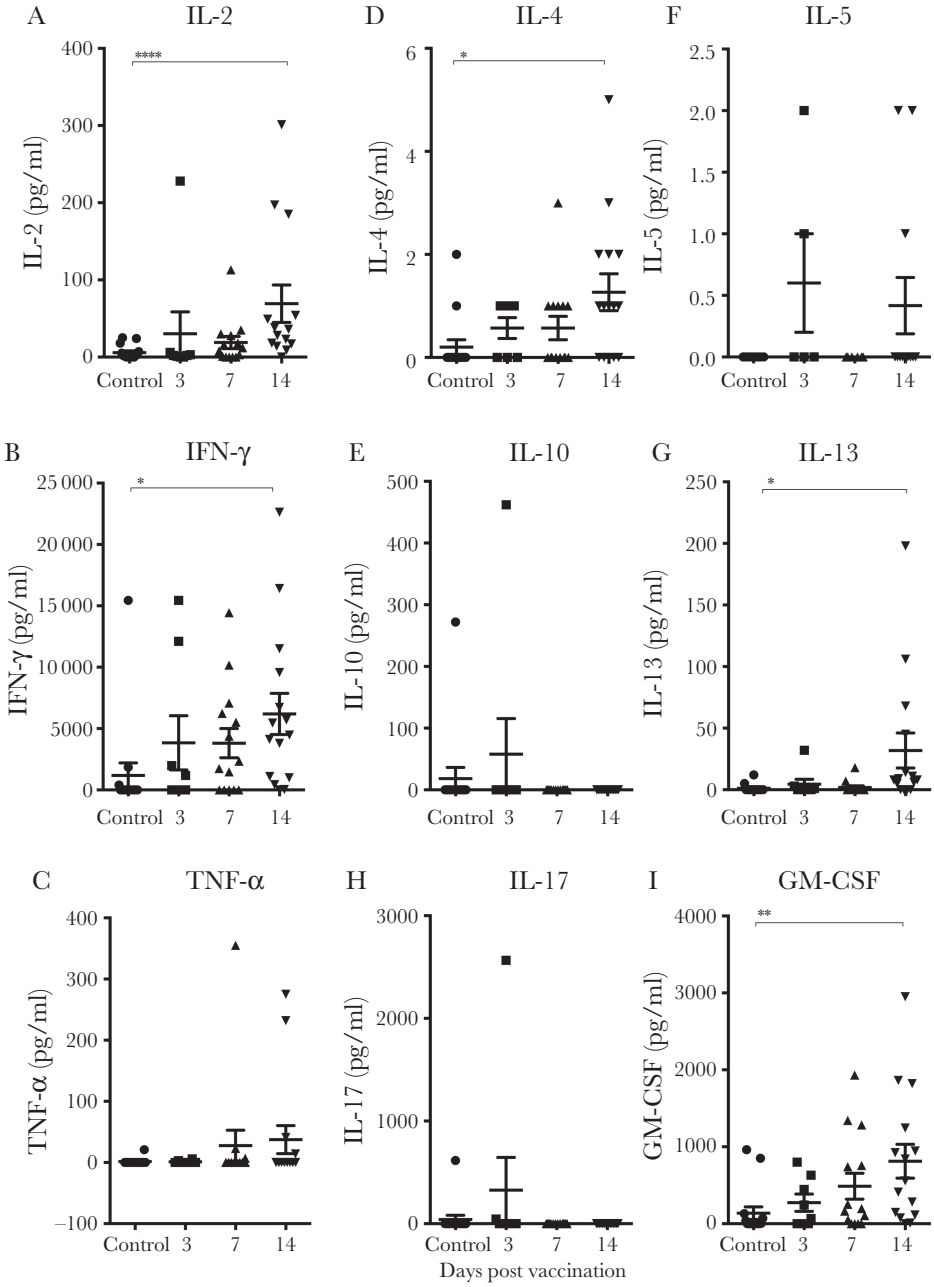


Figure 5. Cytokine responses after live-attenuated influenza vaccination (LAIV) in tonsillar mononuclear cells (TMCs). The TMCs were isolated from nonvaccinated controls and from vaccinees at 3, 7, and 14 days after immunization with LAIV. The TMCs were stimulated for 72 hours with a mixture of split virus antigens from influenza A H1N1, influenza A H3N2, and B vaccine strains, and supernatants were analyzed by multiplex for the presence of cytokines. The Th1 (interleukin [IL]-2 [A], interferon [IFN]-γ [B], and tumor necrosis factor [TNF]-α [C]), Th2 (IL-4 [D], IL-5 [E], IL-10 [F], and IL-13 [G]), and Th17 (IL-17 [H], granulocyte-macrophage colony-stimulating factor [GM-CSF] [I]). Each symbol shows the influenza-specific cytokine response of 1 subject, and the horizontal lines represent the mean ± standard error of the mean. Statistical significance between the cytokine responses in nonvaccinated controls and vaccinated subjects was determined by the non-parametric Kruskal-Wallis multiple comparisons test. *, $P < .01$.

The use of conserved peptide epitope panels provides an important tool to evaluate cross-protective, T-cell responses after LAIV vaccination. Due to the high conservancy score of the universal epitopes used in this study, we conclude that LAIV vaccination of children can induce cross-reactive mucosal T-cell responses that cover a wide range of seasonal and potential pandemic strains. This important knowledge supports the use of LAIV in this age group to elicit cross-protective cellular immune responses. Together with our previous findings of systemic $CD8^+$ T cells, this new knowledge indicates a broader protective immune response after LAIV than previously acknowledged. These findings might provide the immunological basis to explain the protection observed in the absence of protective HI antibodies (HI) and the lower hospitalization rates after childhood LAIV vaccination in the United Kingdom [41, 42].

The HI is commonly used as a COP when evaluating inactivated vaccines. However, induction of HI antibodies after mucosal LAIV does not sufficiently reflect the immune responses, due to the compartmentalization of the immune system. A body of experimental data supports the introduction of cellular assays as a relevant correlate of protection, although they are not yet fully accepted by the regulatory authorities for approval of vaccines [43]. A large efficacy trial in children suggested IFN-γ ELISPOT counts of ≥100 SFU/million PBMCs, as a correlate of protection after LAIV. Furthermore, background levels of <20 SFU/million PBMCs have been found in a United Kingdom child cohort [4]. However, prevaccination levels of >100 SFU/million PBMCs were found in our Norwegian cohort [21], probably due to previous infection. The IFN-γ ELISPOT assays may have a great potential as a correlate of protection, but this is laborious in a clinical setting [3, 44, 45]. A consensus has not been reached within the field, and the number of 100 IFN-γ-producing cells is considered arbitrary [3]. Advances in the research field may lead to the development of a more rapid and convenient assay.

In this study, we have used a relatively small cohort of children, but the data shown here are in line with previous published results for LAIV vaccination [46–48]. The ELISPOT analysis was done based on unfractionated tonsil cells, and the distinction made between $CD8^+$ and $CD4^+$ T-cell responses was based on the selective ability of the peptide epitopes used to be presented by major histocompatibility complex class I or II molecules as empirically verified [49]. A low level of additional cross-presentation can nonetheless not be excluded.

Children are the main transmitters of influenza in the community, and, when infected, they shed virus for a longer period compared with adults. Hence, childhood vaccination campaigns could limit the spread of influenza in the community. Indeed, after the United Kingdom commenced LAIV vaccination of children, signs of herd immunity have been observed in areas with widespread vaccination, such as reduced hospital admission of children [42]. In Japan, childhood IIV vaccination was found to have an indirect effect with a reduction in mortality rates in adults [50]. Successful LAIV immunization requires replication of the LAIV viruses in the mucosa of the upper airways to induce protection. Pre-existing antibodies or local cellular immunity could inhibit replication and hence immune response. The LAIV may be most suitable in the youngest children, with a naive immune response, and perhaps subsequent booster vaccinations should be with IIV. This would ensure a broad cellular and humoral response after LAIV, which can be further expanded by IIV immunizations to secure neutralizing immunity [35].

CONCLUSIONS

In this study, we provide the first evidence of LAIV eliciting cross-reactive $CD8^+$ T-cell responses in the tonsils of young children. These T cells have the potential to provide broad protection against seasonal and pandemic viruses, supporting the use of LAIV as a childhood vaccine.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Supplementary Table 1. Set of Conserved CD4o and CD8 or AH1N1pdm09-Specific Epitopes[a]

[a]Peptides covering unique CD4 and CD8 epitopes from the A(H1N1)pdm09 influenza virus. All of these epitopes are conserved in all 4 swine-origin H1N1 strains (A/California/07/2009, A/England/195/2009, A/Mexico-city/004/2009, and A/Paris/2592/2009). Epitopes are listed in the order of their selection by a greedy algorithm (Order). For each epitope, its estimated response frequency from the literature (ie, Prevalence), fraction of strains that contain 100% matches of the epitope (Conservancy), and the fraction of predicted HLA supertype coverage (S-type_coverage) are also listed in the table.

Supplementary Figure 1. Cross-reactive $CD4^+$ and $CD8^+$ T-cell responses to H1N1pdm09 after LAIV vaccination. The T-cell immune response was evaluated by measuring the number of influenza-specific IFN-γ-secreting T cells (spot forming units [SFU]) after LAIV, using the ELISPOT assay. TMCs isolated from tonsils were tested for responses against panels of peptides representing conserved T-cell epitopes of H1N1pdm09 influenza strain (A and B). Low levels of H1N1-specific IFN-γ-secreting TMCs were detected in nonvaccinated controls, and no increase in IFN-γ-secreting TMC response was detected after vaccination. Each symbol represents the influenza-specific SFU per million TMC for each child with the mean and stand error of the mean (SEM) shown. Statistical differences between vaccinated and nonvaccinated subjects were determined by the nonparametric Kruskal-Wallis (*, $P < .05$; **, $P < .005$).

Notes

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