MAJOR ARTICLE

Longevity of B-Cell and T-Cell Responses After Live Attenuated Influenza Vaccination in Children

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Background. The live attenuated influenza vaccine (LAIV) is the preferred vaccine for children, but the mechanisms behind protective immune responses are unclear, and the duration of immunity remains to be elucidated. This study reports on the longevity of B-cell and T-cell responses elicited by the LAIV.

Methods. Thirty-eight children (3–17 years old) were administered seasonal LAIV. Blood samples were collected before vaccination with sequential sampling up to 1 year after vaccination. Humoral responses were evaluated by a hemagglutination inhibition assay, and memory B-cell responses were evaluated by an enzyme-linked immunosorbent spot assay (ELISpot). T-cell responses were evaluated by interferon γ (IFN-γ) ELISpot analysis, and intracellular cytokine staining of $CD4^+$ T cells for detection of IFN-γ, interleukin 2, and tumor necrosis factor α was performed using flow cytometry.

Results. LAIV induced significant increases in B-cell and T-cell responses, which were sustained at least 1 year after vaccination. Strain variations were observed, in which the B strain elicited stronger responses. IFN-γ–expressing T cell counts increased significantly, and remained higher than prevaccination levels 1 year later. Expression of T-helper type 1 intracellular cytokines (interleukin 2, IFN-γ, and tumor necrosis factor α) increased after 1 dose and were boosted after the second dose. Hemagglutination inhibition titers were sustained for 1 year. Vaccine-induced memory B cell counts were significantly increased, and the response persisted for one year.

Conclusions. LAIV elicited B-cell and T-cell responses that persisted for at least 1 year in children. This is a novel finding that will aid future vaccine policy.

Keywords. influenza; LAIV; humoral; T-cellular; longevity; pediatric; IFN-γ.

Annually, influenza virus infection has a large socioeconomic burden on society, with 500 000 fatal cases globally [1–3]. The World Health Organization estimates that 20% of children are infected with influenza virus each year, and they are the main source of spread of the virus and have a high burden of the disease [4]. Vaccination is the cornerstone of prophylaxis and is recommended for high-risk patients. The trivalent inactivated influenza vaccine (TIV) is safe and provides protection but may not be the optimal vaccine for young children, owing to their lack of previous infection. Since 2003, the cold-adapted live attenuated influenza vaccine (LAIV), administered as a nasal spray, has been approved in the United States for individuals aged 2–49 years. The vaccine was licensed in Europe in 2012 (for individuals aged 2–17 years) and was implemented in the British childhood vaccination campaign, beginning in 2013 [5]. In June 2014, the Advisory Committee on Immunization Practices preferentially recommended LAIV in healthy children 2–8 years old when it is immediately available [6].

Received 13 September 2014; accepted 11 November 2014; electronically published 25 November 2014.

Presented in part: Options for the Control of Influenza VIII Conference, Cape Town, South Africa, September 2013; Fifth European Scientific Working Group on Influenza Conference, Riga, Latvia, September 2014,

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The Journal of Infectious Diseases® 2015;211:1541–9

DOI: 10.1093/infdis/jiu654

Despite many years on the market, there are no established correlates of protection for LAIV. Efficacy studies and observational data suggest that the LAIV provides higher levels of protection than TIV in children [7–11]. The hemagglutination inhibition (HI) titer is widely used as a surrogate correlate of protection after TIV receipt; however, the cutoff titer of 1:40 is based on adult trials. There is debate on both the protective HI level in children and the fact that an HI titer underestimates the protection obtained by LAIV [12–14]. Cellular immunity may be a better measure of protective immunity after LAIV in children [15]. $CD4^+$ T cells have the ability to act as effector cells and to direct and generate specific memory $CD4^+$ and $CD8^+$ T-cell subsets with diverse functions [16–18]. Animal and human studies have demonstrated protective cellular immunity after LAIV [10, 19–21]. Therefore, the duration and quality of the immune response in humans after LAIV needs to be studied.

We conducted a pediatric clinical trial to elucidate the immunological mechanisms induced by LAIV, with emphasis on the long-term, strain-specific cellular immune responses. Our study is unique, as we obtained sequential blood samples from young children up to 1 year after vaccination, allowing us to compare and analyze serum and cellular responses in the same child. Our results indicate that LAIV induces long-term humoral and cellular immune responses in children and that priming is important in determining the magnitude of the response.

MATERIALS AND METHODS

Patients and Study Design

Thirty-eight healthy children, consisting of 20 boys and 18 girls aged 3–17 years old, were recruited at Haukeland University Hospital (HUH) in Norway. From October 2012 to January 2013, children were immunized with the trivalent seasonal LAIV (Fluenz, Astra Zeneca, Birmingham, United Kingdom). Fluenz contained $10^{7.0}$ fluorescent focus units of attenuated reassortant of A/California/7/2009(H1N1)pdm09-like, A/Victoria/361/2011(H3N2)-like, and B/Wisconsin/1/2010 strains. The vaccine was administered intranasally as 0.1 mL per nostril. Children 3–9 years old received 2 doses at a 4-week interval, and children ≥10 years old received a single dose of vaccine as recommended by the manufacturer. The study was approved by the Regional Ethical Committee of Western Norway and the Norwegian Medicines Agency and was monitored by HUH (clinical trials registration, NCT01866540; EUDRACT registration 2012-002848-24).

Upon enrollment, parents and children aged ≥12 years provided voluntary, written informed consent. We collected data on baseline demographic characteristics, medical and influenza vaccination history, and risk factors for influenza virus infection. All procedures were conducted at the pediatric trial unit at HUH. Children with mild-to-moderate asthma (clinically stable with daily use of inhalators) or who had received prior influenza vaccination were included. Exclusion criteria are listed in Figure 1. A self-reported questionnaire of local and systemic side effects was completed after each vaccination.

Samples

Blood samples (volume, 8 mL) were collected after vaccination (Figure 1). Fresh peripheral blood mononuclear cells (PBMCs) were isolated using Cell Preparation Tubes (BD, New Jersey) [22]. Plasma samples were aliquoted and stored at −80°C before use in the HI assay.

HI Assay

Plasma samples from each individual were tested in duplicate by means of an HI assay, using 8 hemagglutination units of the

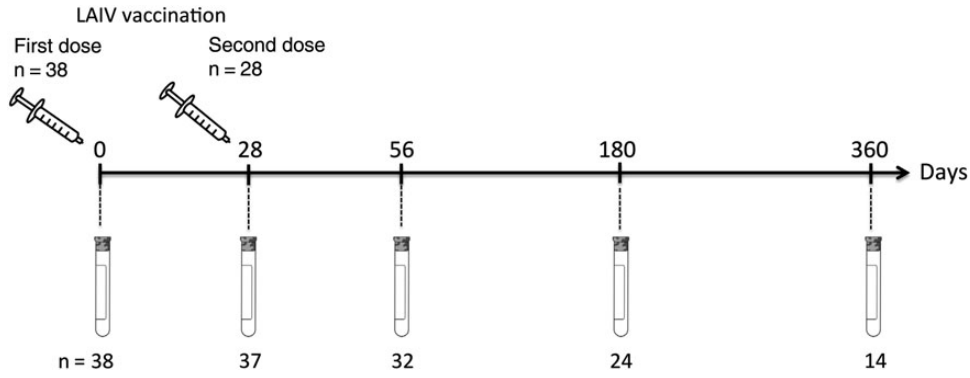

Figure 1. Study design. Healthy children scheduled for elective tonsillectomy were recruited from the Ear, Nose, and Throat Department, Haukeland University Hospital (HUH), Bergen, Norway. Thirty-eight children received live attenuated influenza vaccine (LAIV), and 29 children (all <10 years old) received a second dose. Blood samples were collected before vaccination and at 4 time points after vaccination. The number of subjects providing samples at each time point is shown. Not all children provided blood samples at all visits, owing to difficulty in obtaining blood samples with a sufficient volume (ie, 8 mL). Exclusion criteria were as follows: serious, chronic medical conditions; serious asthma; recent influenza; fever; pregnancy; use of acetyl salicylic acid (ASA) or immunosuppressive therapy; allergy to the vaccine components or earlier complications to vaccination; or under governmental custody.

homologous H1N1 and H3N2 vaccine strains and 0.7% turkey red blood cells [23]. HI titers were defined as the reciprocal of the dilution causing 50% HI. Negative titers were assigned a value of 5 for calculation purposes.

Interferon γ (IFN-γ) Enzyme-Linked Immunosorbent Spot (ELISpot) Assay

IFN-γ–precoated 96-well plates were used according to the manufacturer's instructions (Mabtech, Sweden). PBMCs (400 000 cells/well) in Roswell Park Memorial Institute medium plus 10% fetal calf serum were added to wells, along with negative control (medium alone) or influenza virus antigens (5 μg/mL of split virus vaccine of each strain; H1N1, H3N2, B). Plates were incubated overnight (37°C, 5% CO_2) and developed the following day. The plates were read using the Immunoscan reader and associated software (CTL-Europe). The negative control was subtracted from the influenza virus–specific response.

Intracellular Cytokine Staining (ICS) of $CD4^+$ T Cells

Expression of the influenza virus–specific T-helper type 1 (Th1) cytokines (IFN-γ, interleukin 2 [IL-2], and tumor necrosis factor α {TNF-α}) were measured using ICS of $CD4^+$ T-cells. PBMCs were stimulated overnight with a mixture of the 3 split-virus antigens in the vaccine (H1N1, H3N2 and B; 2.5 μg/mL of each protein) in the presence of brefeldin A, monensin, and anti-CD28 and anti-CD49 antibodies (BD Bioscience, San Jose). After overnight stimulation, cells were stained and analyzed on a BD LSR II flow cytometer for the expression of IFN-γ, IL-2, and TNF-α, as described earlier [24]. The antibodies used are provided in Supplementary Table 1, and the gating strategy is specified in Supplementary Figure 2.

Memory B-Cell Response, Determined by ELISpot

The antigen-specific immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM) memory B-cell responses after vaccination were quantified by ELISpot, as described by Pathirana et al [25] and Crotty et al [26]. Influenza virus–specific immunoglobulin-secreting cells per million PBMCs are presented as percentages of the total IgG, IgA, and IgM responses, respectively.

Statistics

Statistical analyses were performed in SPSS, version 17, and GraphPad Prism, version 5 for Mac (GraphPad Software, San Diego, California). For all statistical tests, a P value of $< .05$ was considered significant. ICS results were compared using t test. For the remaining results, analysis of variance (by the Kruskal–Wallis test) with multiple comparisons testing was used.

RESULTS

Study Subjects

Thirty-eight healthy children, including 6 with asthma, received the LAIV (median age, 4 years). Ten children did not receive a second dose owing to an age of ≥10 years (n = 8), concurrent illness at the time of vaccination (n = 1), and withdrawal from the study (n = 1). Samples were collected before and after vaccination with sampling points as indicated in Figure 1.

Safety and Side Effects

The vaccine was easy to administer and well tolerated. Adverse events were solicited by questionnaires during the initial 7 days after vaccination, and 46% of children reported no side effects after the first dose. Reported side effects were mild and mostly local. Seven children (18%) reported runny/congested nose, and 4 (11%) reported systemic side effects (Supplementary Figure 1). Six children had mild or moderate asthma (clinically stable with daily use of local steroids and β2 agonists), of whom 5 reported no side effects after vaccination and 1 reported transient local side effects. Parents of the asthmatic children did not report asthma exacerbation during the trial. In general, reactions often started 2 days after vaccination and mainly lasted 1–3 days (data not shown). One severe adverse event required consultation but not treatment; this occurred in a healthy 17-year-old girl with nontypical influenza-like illness symptoms of arthralgia. After the second dose, 26 children (90%) reported no side effects, and 3 (10%) reported mostly local side effects (Supplementary Figure 1).

HI Antibody Response Against Influenza Virus A Strains Persists for 1 Year

Figure 2*A* and 2*B* show the HI response to the H1N1 and H3N2 strains before and after LAIV receipt. An HI titer of ≥40 was considered a protective response.

Before vaccination, the majority of children (25 [66%]) had protective antibody titers toward H1N1 (geometric mean titer [GMT], 71; 95% confidence interval {CI}, 40–125). Thirteen children did not have protective HI titers, of whom 9 had no detectable antibody (HI titer, < 10) to the H1N1 virus. An increase in HI titer occurred after the first dose (day 28; GMT, 95; 95% CI, 55–164) and after the second dose (day 56; GMT, 111; 95% CI, 64–194), when 27 subjects (84%) had a protective antibody titer (9% seroconverted). Eighteen subjects had HI titers of ≥40 to the H1N1 virus at 180 days, and 6 subjects had no detectable antibodies. At day 360, 11 of 14 children (79%) had a protective HI level (≥40), of whom 3 seroconverted, but 2 of these children had high prevaccination levels. Two children had no detectable antibodies. Four children without prevaccination antibodies remained seronegative throughout the study.

For the H3N2 strain, 14 (37%) of the 18 children (47%) with an HI titer of < 40 were seronegative (HI titer, 5; GMT, 37; 95% CI, 20–68; Figure 2). After the first dose, there was a significant increase in HI titers ($P < .0001$) in all children except 2, reaching protective HI levels (GMT, 286; 95% CI, 203–401). The increase observed after the second dose was significant, compared with the titer on day 0 ($P < .001$), as well as the titer on day 180 ($P < .01$),

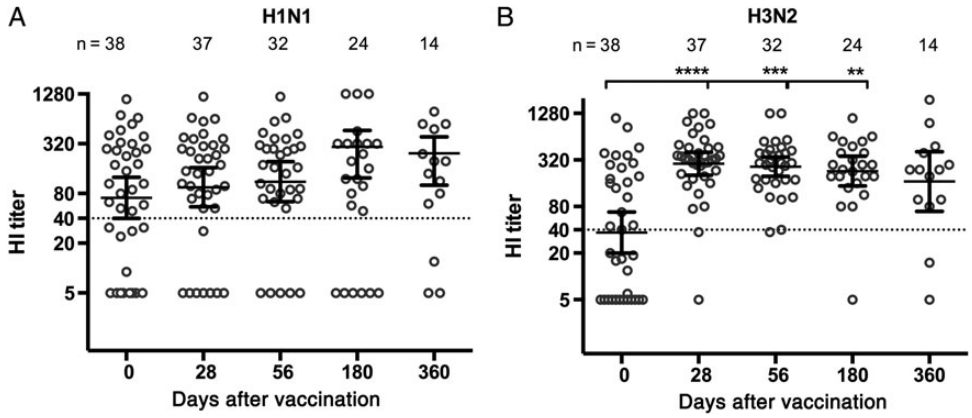

Figure 2. Hemagglutination inhibition (HI) antibody titers after vaccination. Children were intranasally vaccinated with 1 (for those aged ≥10 years) or 2 (for those aged <10 years; doses were administered at a 28-day interval) doses of live attenuated influenza vaccine. HI antibody titers to H1N1 (*A*) and H3N2 (*B*) were measured at the following time points: day 0 (before vaccination), day 28 (after the first dose), day 56 (after the second dose), and days 180 and 360 after vaccination. Each symbol represents the HI response of 1 child, with bold horizontal lines and whiskers denoting geometric mean titers and 95% confidence intervals, respectively. The dotted line represents an HI titer of 40, considered the protective level [27]. The statistical significance of differences from prevaccination levels was determined by analysis of variance, using the nonparametric Kruskal–Wallis test. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

and 47% of the children seroconverted. One 4-year-old child had an HI titer of 40 after 2 doses but had no detectable titers at other time points. The antibody titers remained elevated 180 days after vaccination, with 96% of subjects (n =23) having protective HI titers (GMT, 229; 95% CI, 147–357). At day 360, 12 subjects (86%) had sustained a protective HI antibody response (GMT, 169; 95% CI, 69–410), while the titer in only 2 children remained <40. Of the 14 children evaluated at day 360, 8 (57%) seroconverted. There was no significant difference in the durability of the HI response for either strain in children receiving 1 or 2 doses of vaccine (Supplementary Figure 3).

Long-term Increased IFN-γ Response

We measured the IFN-γ response by using an ELIspot, and we observed interstrain variations. The highest numbers of specific IFN-γ–secreting cells after vaccination were towards the B strain, followed by the H3N2 strain, and the lowest numbers was to the H1N1 strain. Before vaccination, the majority of children (77%) had levels of IFN-γ–secreting T-cells of ≥100 spot-forming cells (SFCs)/10^6 PBMCs that were specific to H1N1, which is a suggested level of protection against influenza (Figure 3*A*) [15]. There was a significant increase in IFN-γ–secreting cells 28 days after the first dose ($P < .05$) and a further increase after the

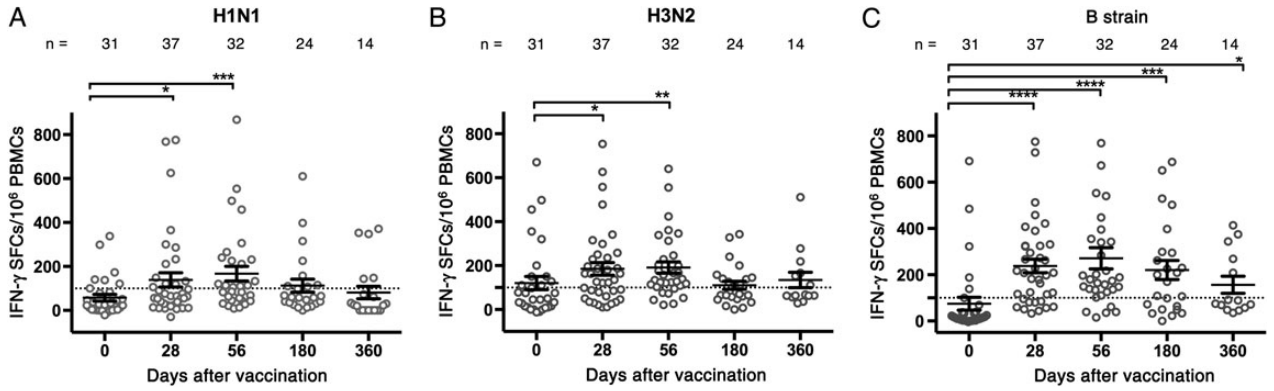

Figure 3. Long-term interferon γ (IFN-γ) immune response in blood after live attenuated influenza vaccination (LAIV). The long-term immune response was evaluated by measuring the number of IFN-γ–producing T cells, measured as spot-forming cells (SFCs)/10^6 peripheral blood mononuclear cells (PBMCs) after LAIV, using the IFN-γ enzyme-linked immunosorbent spot assay. Children were intranasally vaccinated with 1 (for those aged <10 years) or 2 (for those aged ≥10 years; doses were administered at a 28-day interval) doses of LAIV. Blood samples were collected at 0, 28, 56, 180, and 360 days after vaccination. Each symbol represents the influenza virus–specific SFCs/10^6 PBMCs for each child for each influenza strain in the vaccine (*A*, *B*, *C*), with bold horizontal lines and whiskers denoting mean values and standard errors of the mean, respectively. The dotted line represents 100 SFCs/10^6 PBMCs, considered the protective level [15]. The statistical significance of differences from prevaccination levels was determined by analysis of variance, using the nonparametric Kruskal–Wallis test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

second dose ($P < .001$). The levels declined toward day 180, decreasing below the proposed protective level at day 360 after vaccination, but the mean value remained higher than prevaccination levels, although the difference was not significant. Six of the 7 children with ≥100 SFCs before vaccination had received the Pandemrix vaccine.

For the H3N2 strain (Figure 3*B*), 20 subjects (65%) had <100 SFCs/10^6 PBMCs before vaccination, and the numbers of H3N2-specific IFN-γ–secreting cells increased significantly after the first ($P < .05$) and second ($P = .01$) doses. Levels declined toward day 180 and increased slightly again by day 360, remaining above the suggested protective level of 100 SFCs/10^6 PBMCs, although the difference from prevaccination levels was not significant. Nine of the 11 children with ≥100 SFCs/10^6 PBMCs before vaccination had received the Pandemrix vaccine. There was no significant difference in IFN-γ response in children receiving 1 or 2 doses (Supplementary Figure 4)

The response was highest toward the B strain, with 25 subjects (80%) exhibiting <100 SFCs/10^6 PBMCs before vaccination, but it increased significantly after the first dose ($P < .0001$), with a subsequent boost after the second dose ($P < .0001$). By days 180 and 360, the levels declined, but the mean levels remained above the protective level and were significantly higher than at day 0 ($P < .001$ and $P < .05$, respectively).

The IFN-γ response at each time point after vaccination was plotted against the HI response at day 0 (the prevaccination effect) and day 28 (the postvaccination effect) after immunization. There was a significant correlation between the fold increase in HI titer for H1N1 and the fold increase in IFN-γ secretion (Spearman $r = 0.438$; $P = .036$), but the correlation was not observed for H3N2 (data not shown).

Further analysis of the 5 children who remained seronegative (HI titer, 5) to the H1N1 strain after vaccination (2 doses) found a significant increase in influenza virus–specific IFN-γ responses after vaccination ($P = .029$).

Increased Multifunctional CD4⁺ T-Cell Response After LAIV Receipt

Figure 4*A* shows that the frequency of Th1 $CD4^+$ T cells that express a single cytokine (IFN-γ, IL-2, or TNF-α) increased after the first dose and was significantly higher after the second immunization, compared with prevaccination levels. Similarly, the percentage of multifunctional $CD4^+$ T cells expressing either 2 (IFN-γ and IL-2, or IL-2 and TNF-α) or 3 (IFN-γ, IL-2, and TNF-α) cytokines increased after the first dose and significantly increased after the second dose, compared with prevaccination levels (Figure 4*B*).

Increased Long-term Memory B-Cell Response After LAIV Receipt

We evaluated the long-term influenza virus–specific memory B-cell response (IgG^+, IgA^+, and IgM^+) after LAIV receipt (Figure 5).

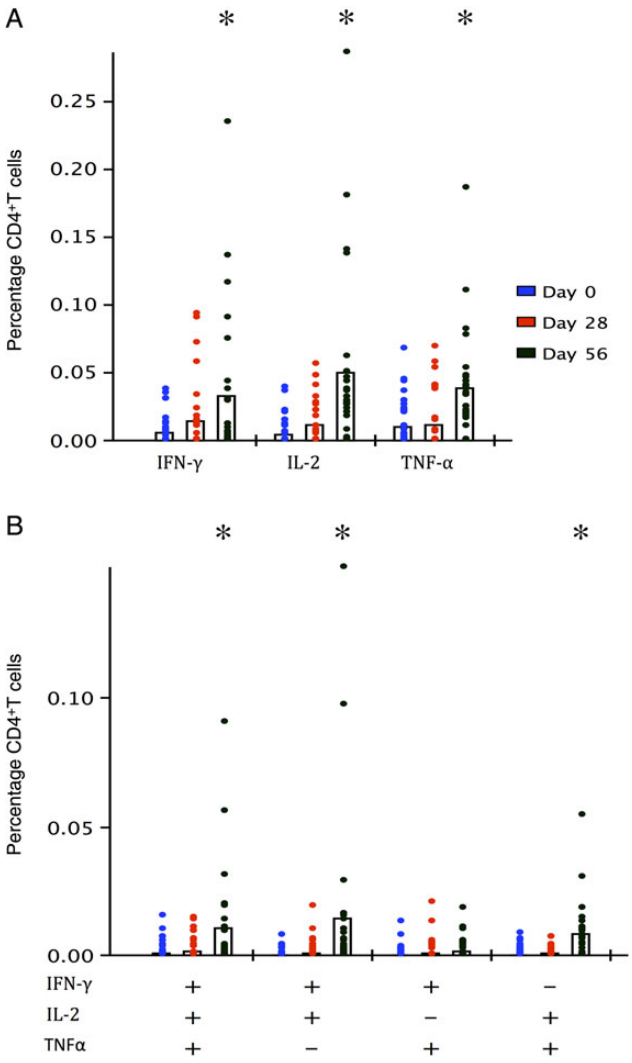

Figure 4. The $CD4^+$ T cell cytokine (T-helper type 1 [Th1]) response before and after vaccination. Peripheral blood mononuclear cells obtained before vaccination (day 0) and 28 and 56 days after vaccination were simulated overnight with split-virus antigen from a mixture of the 3 virus strains in the vaccine (H1N1, H3N2, and B). The percentage of $CD4^+$ T cells secreting either single (*A*) or multiple (*B*) Th1 cytokines was measured by multiparametric flow cytometry. *$P < .05$, by the Student *t* test, compared with the $CD4^+$ T-cell response before vaccination (day 0). Abbreviations: IFN-γ, interferon γ; IL-2, interleukin 2; TNF-α, tumor necrosis factor α.

Overall, the highest frequencies were measured toward the B strain, and the lowest were observed toward the H1N1 strain.

The levels of H1N1-specific IgG^+ memory B cells were high before vaccination and remained elevated at all sampling points after vaccination. The IgM^+ memory B-cell frequencies to H1N1 increased significantly at all time points after vaccination ($P < .01$), whereas the IgA^+ memory B-cell response was only significantly higher on day 360 ($P < .01$).

For the H3N2 strain, IgG^+ memory B cells increased after the first dose of vaccine, with a significant boost ($P < .001$) after the second dose. The frequencies of H3N2-specific IgG^+ memory B

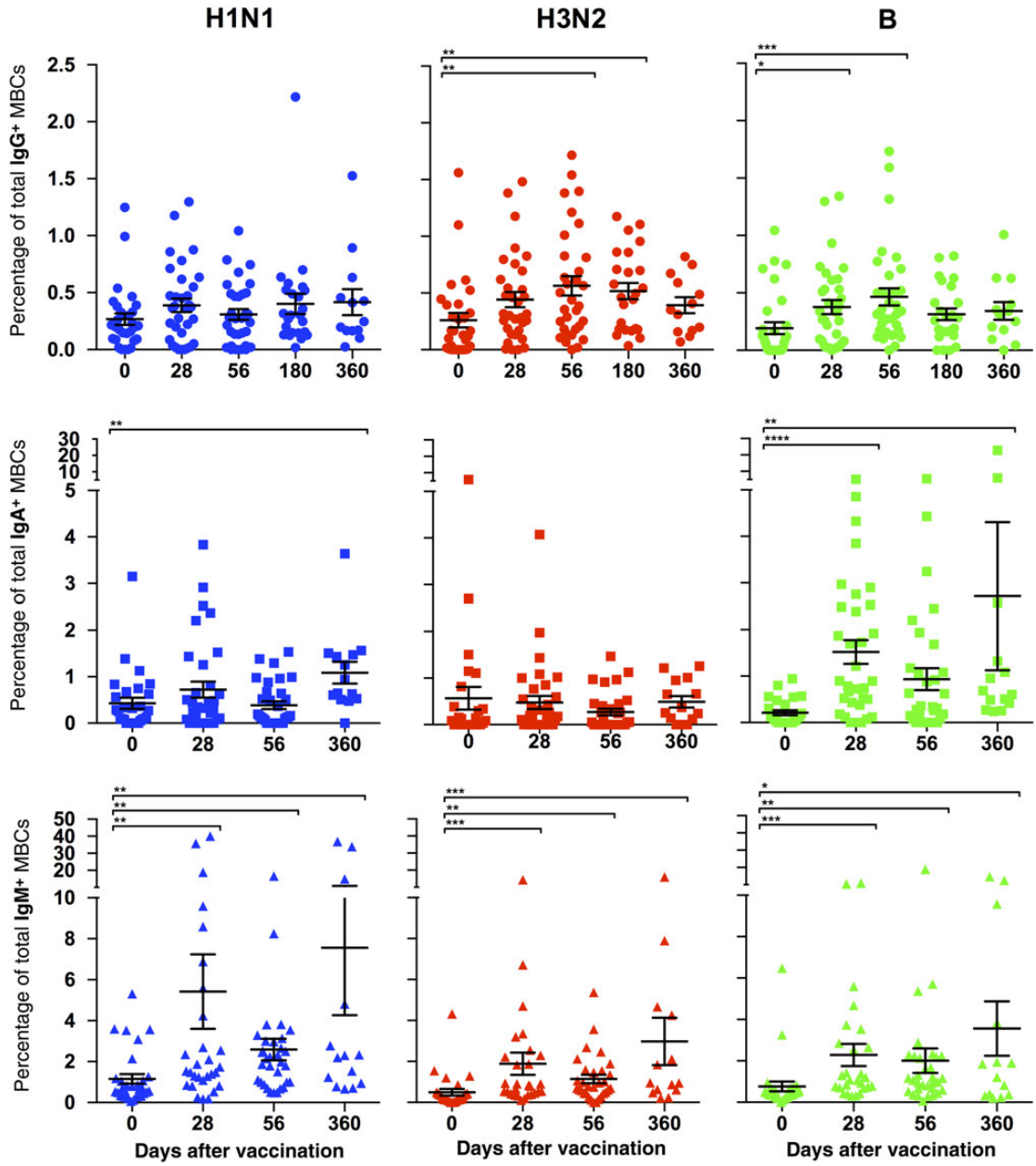

Figure 5. Long-term memory B-cell (MBC) responses after live attenuated influenza vaccination (LAIV). The frequencies of influenza virus–specific immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM)–expressing MBCs before and after LAIV receipt. MBCs were stimulated to proliferate and differentiate into antibody-secreting cells by mitogens in vitro, and levels were subsequently measured by an enzyme-linked immunosorbent spot assay. The *y*-axis shows the percentage of influenza virus–specific MBCs. IgG^+, IgM^+, and IgA^+ MBCs were measured against the 3 influenza virus strains in the vaccine. Data are represented as the percentage of antigen-specific IgG^+, IgA^+, and IgM^+ MBCs among all IgG^+, IgA^+, and IgM^+ MBCs, respectively. Each symbol represents 1 child. IgA^+ and IgM^+ MBC frequencies for day 180 were not determined because of laboratory constraints. The lines represent mean values ± standard errors of the mean. The statistical significance of differences from prevaccination levels was determined by analysis of variance, using the nonparametric Kruskal–Wallis test. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

cells were maintained at day 180 ($P < .01$) and decreased at day 360, although they remained higher than at day 0. We did not detect increased levels of IgA^+ memory B cells to H3N2 at any time point after vaccination; in contrast, IgM^+ memory B cells increased significantly at all time points ($P < .01$).

For influenza B, the frequencies of all immunoglobulin classes of memory B cells increased significantly after 1 dose of vaccine, with *P* values of <.05 for IgG and IgA and <.001 for IgM, for which the highest numbers were observed. After the second immunization, both the IgG^+ and IgM^+ memory B

cells increased significantly ($P < .01$). At day 360, the frequencies remained higher than those observed before vaccination for all 3 immunoglobulins.

DISCUSSION

Clinical pediatric trials with LAIV generally focus on short-term responses after 1 or 2 doses or on efficacy studies performed during influenza seasons [9, 28, 29]. Less is known about the long-term immunological responses, but there are studies reporting the durability of serum and local antibodies and the efficacy of LAIV [30, 31]. The aim of this study was to evaluate the longevity of the humoral and cellular immune responses elicited by LAIV in young children, with a focus on the effect of priming. To our knowledge, our study is unique in reporting the long-term immune responses to LAIV in children, including those with asthma. We found that the vaccine was safe and easy to administer, with mild side effects after the first dose and even fewer side effects after the second dose (Supplementary Figure 1).

This study was conducted during a postpandemic period, when the H1N1 strain dominated. In general, the highest immune response was against the B strain, followed by the H3N2 strain, with the lowest response against the H1N1 strain. The total levels of serum IgG, IgA, and IgM were stable throughout the study period [32]. The serological response to LAIV was evaluated by an HI assay, with differences observed in the response to the H1N1 and H3N2 strains. Before vaccination, the majority of the children had protective antibody titers (HI titer, ≥40) against the H1N1 strain, which did not increase after vaccination but remained elevated 1 year after vaccination. This may be because H1N1 was a dominant circulating influenza A strain in 2009 and 2010. Hence, most of the children were primed, either by natural infection or pandemic vaccination [33]. As LAIV must replicate to elicit an immune response, the presence of preexisting antibodies or cross-reactive T cells in primed children could inhibit virus infection and replication, resulting in lower HI responses. In contrast, the H3N2 strain circulated to a much lesser degree in Norway; hence, most children were unprimed against this strain [33]. The B strain had limited circulation in the prior seasons, except during 2010–2011; it is therefore possible that most children were naive to this strain, consistent with the observation that LAIVs elicit stronger immune responses in unprimed children [34]. Apart from priming, differences in infectivity among the vaccine strains could also impact the subsequent immune response. We saw no difference in durability of the immune response after 1 or 2 doses of vaccine (Supplementary Figures 3 and 4).

Induction of long-term immunological memory is the ultimate goal of vaccination. In this study, levels of influenza virus–specific memory B cells increased after vaccination and were maintained for 1 year. Memory B cells can rapidly differentiate into antibody-secreting plasmablasts upon antigen reencounter. They may possess broad cross-reactivity and the ability to go through secondary affinity maturation to altered antigenic epitopes [35, 36]. The IgG^+ and IgA^+ memory B-cell responses in our study were strain dependent. When we divided the children according to a protective HI titer of 40, we observed that, in influenza virus–primed children, the levels of memory B cells were not boosted upon vaccination (data not shown). The lack of a boosting response after vaccination in B cells in primed individuals has previously been described in adults [37, 38]. In contrast to the IgG^+ and IgA^+ memory B-cell responses, the IgM^+ memory B-cell response increased significantly against all 3 influenza virus strains. Recent research suggests that isotype-switched, affinity-matured memory B cells dominate the antibody-secreting cell response on antigen recall, while the majority of IgM^+ memory B cells contain less somatic hypermutations and dominate the formation of new germinal centers [38, 39]. Furthermore, it has been suggested that IgM^+ memory B cells live longer than their isotype-switched counterparts [39]. Thus, the influenza virus–specific IgM^+ memory B cells may contain a population with the potential to respond to novel antigenic variants (eg, drifted influenza viruses). The observed strain variations may indicate a biological threshold for memory B-cell responses in children with preexisting memory B cells due to previous influenza virus exposure.

LAIV mimics natural infection and activates the innate immune system, as well as both humoral and T-cell responses, which play a key role in cross-reactive anti–influenza virus responses [10, 21]. T cells depend on major histocompatibility complex presentation of viral antigens and, hence, cannot prevent infection per se. Human studies have shown naturally acquired $CD4^+$ and $CD8^+$ T cells to be important in limiting disease and may provide heterosubtypic immunity, which may influence the influenza A (H1N1 and H3N2) response [40, 41]. T cells respond to conserved epitopes, which is why the response to influenza A viruses is interlinked. Importantly, cross-reactive T cells elicited by LAIV have the potential to protect against drifted strains and shifted pandemic strains. This has been demonstrated in animal models [42, 43]. LAIV-induced cross-reactive antibodies have been found in humans, but it has yet to be determined whether LAIV induces cross-protective T cells in humans.

Using a direct ELISpot assay, we determined the IFN-γ T-cell response following LAIV immunization. This ELISpot assay is more sensitive than serum antibody responses in determining the influenza virus–specific memory immune response with an arbitrary number of 100 SFC/10^6 PBMCs suggested as a protective level against clinical influenza after LAIV in a trial of >2000 children [15]. Interestingly, 5 subjects in our study who, on the basis of HI assays, did not seroconvert to H1N1 had a significant increase in their IFN-γ response after 2 vaccine

doses, which may provide clinical protection. In fact, HI titers are known to underestimate the protective effect achieved by the LAIV in children, and clinical efficacy studies on LAIV have shown high levels of protection against laboratory-confirmed influenza despite low HI titers [7, 44, 45]. Studies in adults have found that LAIV elicits higher $CD4^+$ T-cell responses than TIV to the variant region of hemagglutinin, suggesting that antigenically distinct mutants that escape antibody responses may still be recognized by T cells [21].

Recent studies suggest that $CD4^+$ T cells that simultaneously secrete IFN-γ, IL-2, or TNF-α (multifunctional T cells) are functionally superior than single cytokine producers at inducing anti–influenza virus immunity [46]. In this study, we have shown that LAIV induces a significant increase in both single-cytokine and multifunctional Th1 responses in children. The magnitude of the Th1 cytokine responses induced after LAIV receipt was lower than observed in adult subjects after intramuscular vaccination with candidate pandemic vaccines [25, 47]. Differences in the route of administration (intramuscular or intranasal) and formulation with the adjuvant in immunologically naive subjects could partly explain the superiority of the parenteral vaccine at inducing a Th1 response in peripheral blood. However, in children, LAIV has been shown to be a better inducer of T-cell responses than TIV [10, 48, 49]. This may explain, at least in part, the higher efficacy of LAIV, compared with TIV, in children during head-to-head clinical trials [29]. Nonetheless, vaccine-induced long-lived memory $CD4^+$ T cells may provide broader protection and should be a goal of novel vaccines [18]. With respect to cytotoxic T cells, we did not detect an increase in antigen-specific $CD8^+$ T-cell responses at any time point after LAIV receipt. This is most likely due to the use of split virus proteins for in vitro PBMC stimulation, resulting in inefficient antigen cross-presentation to stimulate a $CD8^+$ T-cell response.

Here, we have demonstrated that LAIV elicits elevated and sustained humoral and T-cell responses in young children at least 1 year after vaccination and that there is great interstrain variation in responses. This was recently addressed by the Advisory Committee on Immunization Practices, which noted that the LAIV gave less protection than TIV against the H1N1 strain alone [6]. This study provides support to public health officials in determining the benefit of their childhood vaccine programs when considering safety and obtaining long-lasting immune responses toward influenza virus.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank the children and parents who participated in the study; staff at the Ear, Nose, and Throat Department, Haukeland University Hospital, Dr Lorentz Sandvik, Dr Ole Gamlemshaug, Dr Per Anders Hunderi, Hildegunn Grimstad, Wendela Mathisen, Nina Haugland, staff at the Haukeland University Hospital Pediatric Trial Unit (specifically Hildur Grindheim, Renate Håpoldøy, Anne Marthe Østerbø, and Marianne Heradstveit), and staff at the University of Bergen Influenza Center, for assistance with the clinical trial; Emilia Lohndal, Jane Kristin Nøstbakken, Steinar Sørnes and Dagny Ann Sandness for invaluable technical and logistical assistance; and GlaxoSmithKline, for kindly providing the split virus antigen.

Financial support. This work was supported by the Influenza Center at the University of Bergen and the Bergen Clinical Vaccine Consortium. The Influenza Center is funded by the Ministry of Health and Care Services, Norway, the Norwegian Research Council Globvac program (220670/H10), the European Union (Univax 601738), Helse Vest, and the K. G. Jebsen Center for Influenza Vaccines.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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