Phenotypic Changes in Influenza-Specific CD8+ T Cells after Immunization of Children and Adults with Influenza Vaccines

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The effect of trivalent inactivated influenza vaccine (TIV) or live attenuated influenza vaccine (LAIV) on the phenotypes of circulating influenza-specific CD8+ T cells was analyzed by interferon (IFN)–γ flow cytometry and tetramer staining. In adults, the expression of the T cell differentiation marker CD27 on virus-specific CD8+ T cells decreased after LAIV but increased after TIV. In children, expression of the cytotoxicity molecule perforin in influenza-specific CD8+ T cells increased after TIV but not after LAIV. Among children aged 6 months to 4 years who had not been vaccinated previously and who received 2 doses of TIV, CD27 expression decreased after each dose, whereas perforin expression increased after the second dose. These findings indicate that the phenotypic changes of influenza-specific CD8+ T cells differ depending on the type of vaccine and the age of the vaccinee. These differences are potentially affected by the different routes of vaccination and pathways of antigen presentation for TIV and LAIV.

Influenza viruses are major respiratory tract pathogens for people of all ages, especially the elderly and very young [1]. Two types of influenza vaccines are currently available: the trivalent inactivated influenza vaccine (TIV), which is given intramuscularly [2], and the live attenuated influenza vaccine (LAIV), which is administered intranasally [3]. TIV is approved for use in people aged 6 months or older. LAIV is currently approved for use in those aged 2 to 49 years. It has been reported that LAIV is more effective than TIV in children with specific medical conditions [4, 5]. A recent study found that, in healthy children aged 6 months to 4 years, LAIV had significantly better efficacy than TIV for both antigenically well-matched and drifted strains [6]. In contrast, it was recently reported that, in healthy adults aged 18 to 49 years, TIV and LAIV were similarly effective against drifted type A (H3N2) viruses, but TIV was superior against type B infections [7]. The underlying mechanisms for the differences in efficacy in children and adults immunized with LAIV versus TIV are not clear.

Antibodies to the influenza virus surface proteins hemagglutinin and neuraminidase have been associated with protection from disease or viral replication after natural influenza infection or vaccination in adults and children [8–14]. Primarily on the basis of findings in animal models, T cell responses are also thought to play an important role in clearing influenza virus infection [15–21]. However, few studies of cellular immunity against influenza have been done in humans, especially in children given LAIV [22–24].

The T cell immune responses to viral infection is a highly dynamic process that involves both quantitative and qualitative changes in virus-specific T cells and their migration between different sites in the body. On exposure or reexposure to viral antigens, naïve T cells or central memory T cells are activated and proliferate in the
local lymph nodes. The activated T cells differentiate into effector memory T cells, which recirculate into the infected tissue and exert effector functions to control the infection [25]. Several T cell markers have been related to different developmental stages of T cells. CD27 is a costimulatory receptor required for the generation and maintenance of T cell immunity [26]. Expression of CD27 has been associated with naive and memory CD8+ T cells, whereas loss of CD27 expression has been associated with effector CD8+ T cells, which are believed to represent a later stage of T cell differentiation [27, 28]. Perforin is another marker associated with CD8+ T cell differentiation. It is one of the most important effector molecules mediating cytotoxic activity of CD8+ T cells [29]. Studies in mouse models have shown that perforin-mediated target cell destruction is a major mechanism by which effector CD8+ T cells terminate influenza infection [30]. The effect of influenza vaccination on the expression of CD27 and perforin on specific CD8+ T cells in humans has not been studied systematically.

During the 2004–2005 influenza season, we studied the NK and T cell responses in children and adults receiving LAIV or TIV [31]. An interferon (IFN)–γ flow cytometric assay was used to detect influenza A virus (fluA)–specific CD4+ and CD8+ T cells in peripheral blood mononuclear cells (PBMCs) exposed to live fluA ex vivo [32, 33]. In addition to assessing the percentages of IFN-γ+ cells in T and NK cell subsets [31], this polychromatic flow cytometric assay allowed simultaneous analysis of phenotypic markers of fluA-specific CD8+ T cells, including CD27 and perforin. The goal of the present study was to investigate the effect of LAIV and TIV on the differentiation of fluA-specific CD8+ T cells by examining their expression of these 2 phenotypic markers before and after vaccination. Our hypothesis was that vaccination affects the characteristics of fluA-specific CD8+ T cells beyond increasing their numbers.

MATERIALS AND METHODS

Human subjects and vaccination protocols. Before the 2004–2005 influenza season, 26 children aged 6 months to 4 years, 39 children aged 5 to 9 years, and 44 adults aged 22 to 49 years were enrolled into a multiproject influenza vaccine study during the period of 23 September to 1 December 2004. The study protocol was approved by the institutional review board at Stanford University. Informed consent was obtained from subjects or their parents, and assent was obtained from children ≥7 years old. Details of the demographic information on the study subjects and the vaccination and blood sampling protocol for each age group have been published elsewhere [31].

Subjects were immunized with either TIV (Fluzone; Aventis Pasteur) or LAIV (FluMist; MedImmune) according to guidelines for influenza vaccination at the time of the study. All of the adults had been randomized to receive 1 dose of either TIV or LAIV for the previous 2003–2004 influenza season. For the present study, each adult was revaccinated with 1 dose of the same type of vaccine. Children aged 5 to 9 years were randomized 1:1 to receive either TIV or LAIV. Three blood samples were collected from the adults and children on day 0, approximately day 10, and day 28–42 after vaccination. Children in the youngest age group (6 months to 4 years) had not been vaccinated previously and therefore received 2 doses of TIV approximately 28 days apart. LAIV was not approved for this age group at the time of study. Two blood samples were scheduled to be collected from each of the subjects aged 6 months to 4 years, the first before vaccination and the second on approximately day 10 after either the first or second vaccine dose, depending on random assignment. The 2004–2005 influenza vaccines contained influenza A/New Caledonia (H1N1), A/Wyoming (H3N2), and B/Jiangsu (TIV) or B/Jilin (LAIV) strains.

IFN-γ flow cytometry. Freshly prepared PBMCs were incubated with or without purified live fluA/Wyoming strain for 17 h, with brefeldin A (Sigma-Aldrich) added during the last 4 h, as described elsewhere [31]. The cells were stained with Alexa 700–labeled anti-CD27, allophtocyanin (APC)–Cy7–labeled anti-CD8, and phycoerythrin (PE)–Cy7–labeled anti-CD56 (BD Pharmingen); treated with FACS Lysing Solution and FACS Permeabilizing Solution (BD Biosciences); and then stained with a mixture of the following antibodies: PE-labeled anti–IFN-γ, fluorescein isothiocyanate (FITC)–labeled anti-perforin, and peridinin-chlorophyll protein (PerCP)–labeled anti-CD3 (BD Biosciences). Stained cells were analyzed with an LSR II flow cytometer (BD Biosciences).

Tetramer staining. Frozen PBMCs were thawed, washed, and typed for HLA-A2 with monoclonal antibodies MA2.1 and BB7.2, as described elsewhere [34]. HLA-A2+ samples were stained with PerCP-labeled anti-CD8, APC-Cy7–labeled anti-CD4 (BD Pharmingen), Alexa 700–labeled anti-CD27, and PE-labeled iTAg MHC tetramer with influenza M1 peptide GILGFVF (Beckman Coulter). The stained cells were treated with FACS Lysing Solution and FACS Permeabilizing Solution and then stained with FITC-labeled anti-perforin. Stained cells were analyzed with an LSR II flow cytometer.

Statistical analysis. Data are summarized descriptively throughout as means ± 1 SE. Correlations were estimated using Spearman’s coefficient. Two-sample comparisons were made using Student’s t test. Repeated-measures data were analyzed using general linear models for longitudinal data [35] or mixed-model regression analysis [36], the choice depending on which model’s assumptions were most closely met by the data. Type I error rate was controlled at 5% within each figure by use of sequential Bonferroni adjustment [37].

RESULTS

Phenotypes of fluA-specific CD8+ T cells before vaccination. Two assays were used to investigate the CD27 and perforin pheno-
types of fluA-specific CD8+ T cells. For all subjects, PBMCs were cultured with live fluA/Wyoming (H3N2) for 17 h and then analyzed with IFN-γ flow cytometric assay to detect fluA-specific CD8+ T cells and to determine their expression of CD27 and perforin. For the HLA-A2–positive adults, uncultured PBMCs were also directly stained with M1 tetramer, along with surface staining for CD27 and intracellular staining for perforin. Children were not tested with tetramer due to the smaller volume of blood samples. The estimated correlation between the percentage of M1 tetramer+CD8+ T cells, which represents CD8+ T cells specific for a single epitope of the M1 protein, and percentage of fluA-specific IFN-γ’CD8+ T cells, which represents CD8+ T cells reactive to multiple fluA epitopes, was modest in size yet statistically significant at baseline (day 0) (r = 0.618; P = .006).

At baseline, the average percentage of fluA-specific IFN-γ’CD8+ T cells was significantly higher in the adults than in the children (figure 1A, upper panel). In contrast, the percentage of these cells expressing CD27 or perforin was significantly higher in the children than in the adults (figure 1B and 1C, upper panels). Within the group of children aged 6 months to 9 years, a modest positive correlation was observed between age and percentage of IFN-γ’CD8+ T cells at baseline (figure 1A, lower panel). A modest negative correlation was observed between age and percentage of cells expressing perforin (figure 1C, lower panel). Taken together, these results show that, when subjects of all ages are considered, the prevaccination frequency of fluA-specific CD8+ T cells increases with age but the level of these cells expressing CD27 or perforin decreases with age, suggesting that age is a factor affecting the phenotype of virus-specific memory CD8+ T cells.

**Phenotypes of fluA-specific CD8+ T cells after LAIV and TIV administration.** To examine whether vaccination induced changes in CD27 and perforin expression on fluA-specific CD8+ T cells, phenotypic analyses were performed along with the quantification of fluA-specific T cells at 2 time points after vaccination. Among adult LAIV recipients, the mean percentage of fluA-specific IFN-γ’CD8+ T cells expressing CD27 was significantly lower than the baseline level on day 10 but not day 28; in contrast, the mean percentage of these cells that expressed CD27 was significantly higher than at baseline on both days 10 and 28 in adult TIV recipients (figure 2A). The difference between the effects of LAIV and TIV, described as the change in the percentage of CD27+ cells after vaccination from their level on day 0, was significant at both time points (figure 2B). In contrast to the findings in adults, there were no significant differences among 5–9-year-old children between the pre- and postvaccination mean percentages of CD27+ cells (figure 2A) or between the effects of LAIV and TIV (figure 2B). The mean baseline CD27
expression was significantly lower in the adult TIV group than in the adult LAIV group (P < .001), but no such difference was detected in children between TIV and LAIV recipients (P = .469). Of note, each of the adult subjects had received a dose of the same type of vaccine during the prior influenza season (2003–2004), whereas the children were randomized to receive 1 of the 2 vaccines during the present study.

In contrast to CD27, neither LAIV nor TIV induced significant changes in the mean percentages of perforin IFN-γ CD8+ T cells among the adults (figure 2C). There was no significant difference between the effects of these 2 vaccines (figure 2D).

However, in children aged 5 to 9 years, the mean percentage of perforin IFN-γ CD8+ T cells increased significantly by day 28 after TIV but not after LAIV (figure 2C), and the difference between the effects of these 2 vaccines was significant (figure 2D).

PBMCs from all HLA-A2-positive adults receiving LAIV were analyzed with the influenza M1 tetramer (figure 3). In agreement with the results of IFN-γ flow cytometry, a significant decrease in the mean percentage of M1-specific CD8+ T cells expressing CD27 was observed on day 10 after LAIV vaccination.
but not on day 28. No significant change was observed in the mean percentage of M1-specific CD8\(^+\) T cells expressing perforin.

**Phenotype of fluA-specific CD8\(^+\) T cells in children aged 6 months to 4 years after TIV vaccination.** Children aged 6 months to 4 years who had not previously been immunized with influenza vaccine received 2 doses of TIV at a 28-day interval. These subjects were randomized at a 1:1 ratio to have blood sampled at 10 days after either the first or second dose of TIV (figure 4). Compared with day 0, the mean percentage of IFN-\(\gamma\)-CD8\(^+\) T cells expressing CD27 was significantly lower on day 10 after the first and second dose of TIV, whereas the mean percentage of IFN-\(\gamma\)-CD8\(^+\) T cells expressing perforin was significantly higher after the second dose but not after the first dose.

**Effects of age on phenotypic change in fluA-specific CD8\(^+\) T cells after TIV vaccination.** To explore the effects of age on the phenotype of fluA-specific IFN-\(\gamma\)-CD8\(^+\) T cells before and after TIV immunization, the percentages of IFN-\(\gamma\)-CD8\(^+\) T cells expressing CD27 at baseline (day 0) and 10 days after the first dose of TIV for all study subjects were regressed on their ages (figure 5A). Because the regression analysis used age as a continuous variable, hypotheses were tested at the median age for each age group (3, 7, and 30 years). The estimated mean percentage of CD27\(^+\)IFN-\(\gamma\)-CD8\(^+\) T cells detected at baseline decreased significantly from age 3 to age 7 to age 30 years (figure 5B). At day 10 after the first dose, the mean change in the percentage of CD27\(^+\)IFN-\(\gamma\)-CD8\(^+\) T cells differed significantly from age 3 to age 7 to age 30 years; children had a mean decrease, and adults had a mean increase (figure 5C). No significant difference was seen in the change in perforin expression in IFN-\(\gamma\)-CD8\(^+\) T cells from TIV vaccinees of different ages (data not shown).

**DISCUSSION**

Previously we reported that TIV and LAIV induced quantitatively different T cell responses in children and adults with regard to the proportion of fluA-specific T cell populations [31]. In the present study, we have demonstrated that these vaccines also induced different changes in the phenotypes of fluA-specific CD8\(^+\) T cells. This assessment of phenotypic changes in fluA-specific CD8\(^+\) T cells, along with the quantitative changes in the number of these cells, provides a more complete picture of the fluA-specific CD8\(^+\) T cell responses elicited by live or inactivated influenza vaccines. Table 1 summarizes the average percentage of fluA-specific IFN-\(\gamma\)-CD8\(^+\) T cells [31] and their CD27 and perforin phenotypes in each age group before and after immunization with LAIV or TIV.

In children in the youngest age group (6 months to 4 years), who had not been vaccinated before and received 2 doses of TIV,
a significant increase in the percentage of fluA-specific IFN-γ+CD8+ T cells was accompanied by decreased expression of CD27 and increased expression of perforin, phenotypic changes consistent with an effector T cell response. Unexpectedly, similar changes were not observed in all age groups receiving TIV or LAIV. In children aged 5 to 9 years, LAIV, but not TIV, elicited a significant increase in the mean percentage of circulating fluA-specific CD8+ T cells. Regarding their phenotype, TIV, but not LAIV, induced a significant increase in the expression of perforin by day 28 after vaccination. In adults, neither TIV nor LAIV induced a significant change in the mean percentage of circulating fluA-specific IFN-γ+CD8+ T cells; however, their expression of CD27 decreased significantly by day 10 after LAIV but increased after TIV administration, indicating that both vac-
cines affected the CD8+ T cell compartment even though the detected frequencies of fluA-specific CD8+ T cells did not change significantly. This finding suggests that vaccination can have more subtle qualitative effects on cellular immunity even in the absence of readily detectable quantitative changes.

It should be mentioned that although CD27 expression on fluA-specific IFN-γ CD8+ T cells increased at both days 10 and 28 in the adult TIV recipients, we believe that this increase was transient. All of the adult vaccinees in this study had received the same vaccine in the previous year. At baseline (day 0) in the present study, the TIV group had a significantly lower level of CD27 expression on their fluA-specific CD8+ T cells than did the LAIV group, suggesting that the expression of CD27 on circulating fluA-specific CD8+ T cells declined later than day 28 after the previous immunization with TIV. Taken together, these findings reveal highly variable patterns in both the quantitative and phenotypic changes in circulating fluA-specific CD8+ T cells after TIV or LAIV administration in healthy children and adults.

Phenotypic changes in virus-specific CD8+ T cells over the course of natural viral infection have been reported in limited studies. In patients acutely infected with parvovirus B19 [38], B19-specific CD8+ T cells had increased perforin expression and decreased CD27 expression after infection. In contrast, subjects who had been infected at remote times had low levels of perforin expression in virus-specific T cells. These findings suggest that down-regulation of CD27 and up-regulation of perforin are associated with a recent encounter of virus-specific T cells with the cognate viral antigen. In agreement with these findings, we have shown that memory CD8+ T cells specific for fluA, which causes periodic self-limited infections, expressed higher levels of CD27 than CD8+ T cells specific for cytomegalovirus, which causes persistent infection, in the same individuals [32]. In addition, in a patient with acute HCV infection who cleared the virus, the percentage of HCV-specific CD8+ T cells and the percentage of such cells expressing perforin declined steadily during a period of 8 months after elimination of HCV (X.-S.H. et al., unpublished data). These observations suggest that the CD27- and perforin+ phenotypes are associated with virus-specific CD8+ T cells that have effector functions, but not with memory CD8+ T cells that have not recently encountered viral antigens. Of note, we showed that fluA-specific CD8+ T cells at baseline before vaccination for the next flu season had higher levels of both perforin and CD27 expression in the children than in the adults, suggesting that the relationship between the exposure history and different T cell phenotypic markers could vary among viruses and age groups.

LAIV and TIV are administered through different routes and elicit CD8+ T cell responses through different antigen presentation pathways. LAIV is administered to the nasal mucosal surface. Similar to natural infection with wild-type fluA, the live attenuated influenza virus is presumed to undergo limited replication at the site of infection and infect antigen-presenting cells, which presents the endogenously synthesized viral proteins to naive or memory CD8+ T cells via proteosome-mediated class I pathways. The differentiated effector memory T cells with down-regulated CD27 expression will eventually migrate back to the infected site, which is the respiratory tract in the case of influenza infection. Consistent with this model, it has been reported that influenza-specific CD8+ T cells with a relatively late differentiation phenotype (CD27low) were enriched in human lungs compared with PBMCs [39]. In contrast, TIV is administered by intramuscular injection, which induces systemic immune responses. The viral protein antigens in this vaccine are derived from inactivated virus and are thought to be presented to CD8+ T cells via the cross-presentation pathway of antigen-presenting cells [40]. Hence, the variable microenvironment in T cell activation sites and different pathways of antigen presentation associated with these 2 vaccines may result in different kinetics and programs of memory T cell differentiation. Of particular interest, it was reported that, after mucosal or systemic immunization, the expression of chemokine receptors on circulating antigen-specific T cells [41] or antigen-specific antibody-secreting cells [42–46] differed according to the route of vaccine delivery, suggesting distinct homing potentials of antigen-specific lymphocytes induced by different types of vaccination.

It should also be emphasized that only 1%–2% of total T cells in the entire body are present in the periphery. Therefore, influenza-specific CD8+ T cells detected in postvaccination blood samples represent only a small subset of the overall influenza-specific CD8+ T cell population in circulation at the time of sampling, and the frequency and phenotypes of such cells may differ from those in the lymphoid organs or infected tissues [39]. Taken together, all of these factors are likely to affect the pattern and kinetics of phenotypic changes in circulating CD8+ T cells detected at certain time points after immunization with different type of vaccines, as shown in the present study. For example, in adults the opposite changes in frequency of circulating CD27+ fluA-specific CD8+ T cells after LAIV and TIV immunization could reflect differences in the efficiency of these vaccines in inducing CD27+ effector T cells and/or in the homing potential of CD27- effector T cells induced by the 2 vaccines. In addition, the phenotypic as well as the quantitative characteristics of T cell responses may depend on the prior virus or vaccine exposure history [46] and the age of vaccinees, as shown in the different changes in CD27 and perforin phenotypes in child and adult TIV recipients.

It is reasonable to hypothesize that phenotypic characteristics of fluA-specific T cells induced by LAIV or TIV are associated with T cell functions and their role in protective immunity. Future studies should focus on further characterizing these phenotypic differences in virus-specific CD8+ T cells induced at different ages by the 2 influenza vaccines and on determining the functional consequences of these phenotypically distinct CD8+ T cell responses.
T cell populations for protective immunity. It will also be interesting to carry out multiyear longitudinal studies detailing the kinetics of phenotypic changes of virus-specific T cells. Such studies may provide insight into the variable efficacy of influenza vaccines and their mechanisms of protective immunity in different age groups.

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References