B- and T-cell memory elicited by a seasonal live attenuated reassortant influenza vaccine: assessment of local antibody avidity and virus-specific memory T-cells using trogocytosis-based method

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Purpose The main purpose of vaccination is to generate immunological memory providing enhanced immune responses against infectious pathogens. The standard and most commonly used assay for influenza vaccine immunogenicity evaluation is a hemagglutination inhibition assay (HAI). It is clear now that HAI assay is unable to properly assess the proven protective immunity elicited by live attenuated influenza vaccines (LAIV). New methods need to be developed for more accurate LAIV immunogenicity assessment and prediction of vaccine efficacy among target populations.

Objective Randomized placebo-controlled study of memory B- and T-cell responses to intranasal LAIV in young adults.

Methods A total of 56 healthy young adults 18–20 years old received seasonal monovalent LAIV. Mucosal memory B-cell responses were measured by IgA avidity assessment in nasal swabs. CD4 memory T cells in peripheral blood were examined by the expression of CD45RO marker and in functional test by the ability of virus-specific T cells to maintain the trogocytosis with antigen-loaded target cells.

Results Intranasal LAIV immunization enhances mucosal IgA avidity even without reliable increases in antibody titers. At the day 21 after vaccination, up to 40% of subjects demonstrated significant increases in both total and virus-specific CD4 memory T cells that were observed regardless of seroconversion rate measured by HAI assay.

Conclusion The data suggest that immunogenicity of LAIV vaccines should be evaluated on the mucosal and cellular immunity basis. The assays applied could be used to support influenza clinical trials through preliminary screening of volunteers and subsequent measurement of anti-influenza in immunity.

Keywords Antibody avidity, immunological memory, live influenza vaccine, trogocytosis.

Introduction

Effective influenza vaccines need to satisfy three major criteria: safety, immunogenicity, and efficacy. According to the regulatory documentation in Russia, the hemagglutination inhibition (HAI) assay remains the only test recommended for evaluation of LAIV immunogenicity. However, the goal of vaccination is not only the induction of serum antibodies, but also the generation of effective immunological memory for long-term protection. In recent years, substantial research efforts have been devoted toward vaccine assessment methods that measure memory cell responses to a wide range of pathogens and cancers.[1–5] In the case of vaccines delivered to mucosal surfaces, development of fast, easy, and cost-effective assays for the measurement of immunological memory continues to be a challenge because of the different nature of the immunity elicited by such vaccines. In this report, an analysis of the LAIV ability to stimulate B- and T-cell immunological memory was performed not only to demonstrate the benefit of this vaccine but also to refine the appropriate immunological assays for comprehensive measurement of live vaccine immunogenicity.

There are several approaches for memory B-cell assessment: (i) by IgM to IgG switching of antibody-producing plasma cells;[6] (ii) B-cell phenotype changing to

$CD27^+$ $CD38^+$ IgD^- $Bcl-2^+$ subpopulation;[4] and (iii) increases in serum antibody avidity.[7–10] Here, we report the modification of the latter approach for the measurement of local IgA avidity in human nasal swabs.

Effective anti-influenza immunity depends on the induction of T-cell immunological memory as well as B-cell immune responses.[2,4,5] Previously, the ability of inactivated influenza vaccine to induce $CD4^+$ $CD45RO^+$ and $CD8^+$ $CD45RO^+$ cells in volunteers was demonstrated without determination of virus-specific cells.[11] Later, the production of influenza-specific IFNγ-producing CD4 and CD8 T cells was studied upon vaccination with inactivated or live influenza vaccines.[12] In our study, we modified the [T-cell recognition of antigen-presenting cells (TRAP) by protein capture] assay that measures antigen-activated cells involved in trogocytosis, i.e., membrane fragments interchange between T cells and APCs during antigen presentation.[13] The TRAP method has been previously used to study trogocytosis *in vitro*[14] and to determine *in vivo* T cells specific for herpes virus,[15] lymphocytic choriomeningitis virus,[16] and ovalbumin.[14]

Materials and methods

Volunteers and vaccination

Participants were 18–20 years old adults without contraindication to LAIV vaccination. After desirable sizes of groups were chosen, persons were randomly (by chance) assigned to receive LAIV or placebo.

Avidity of local IgA antibodies was observed in 56 volunteers vaccinated with attenuated reassortant strain for LAIV A/17/Solomon Islands/06/9 (H1N1) at a dose of 10^8 $EID_{50}/0.2$ ml and 21 volunteers inoculated with placebo (sterile physiological solution). *Levels of $CD4^+$ $CD45RO^+$ cells* were measured in 32 volunteers vaccinated with trivalent LAIV [A/17/New Caledonia/99/145 (H1N1) at a dose of 10^8 $EID_{50}/0.2$ ml, A/17/California/04/6 (H3N2) at a dose of 10^7 $EID_{50}/0.2$ ml, and B/60/Jilin/01/1 at a dose of $10^{6.33}$ $EID_{50}/0.2$ ml] and 25 volunteers inoculated with placebo. *Virus-specific memory T cells* were studied in 20 volunteers vaccinated with attenuated reassortant strain for LAIV A/17/Solomon Islands/06/9 (H1N1) and 10 volunteers inoculated with placebo. Vaccines or placebo were intranasally administered once, 0.25 ml per nostril. Physical examination and venous blood collection were performed before vaccination and 1 month after vaccination. Nasal swab samples were collected before and 21 days after vaccination (day 0 and day 21, respectively).

Nasal swab sample collection

Dry cotton buds were inserted in the volunteer's nostrils along the lateral nasal walls 2–3 cm inside, to inferior nasal conches (volunteer should sit in a comfortable position with the head slightly tilted backward). Cotton buds remained in the nasal passages for 5 minutes. Then, they were slowly withdrawn with three rotating motions. The bud tips were put into a plastic vial containing 0.5 ml of sterile PBS. After 2-hour incubation at 4°C, cotton buds were pulled out of vial and placed into standard 1-ml pipette tip that was further inserted back into the vial and centrifuged for 10 minutes, 200 *g*, +4°C. After centrifugation, the tip with cotton buds was thrown away. Vial containing ready nasal swab sample was stored at −70°C until analysis (but no longer than 3 months).

Separation of human peripheral blood mononuclear cells (PBMC) was carried out by the standard methods[17] using Histopaque-1077 (Sigma-Aldrich Co., St. Louis, MO, USA) separation medium.

Immunogenicity of LAIV was assessed in standard HAI assay using 4 HAU of appropriate viruses. Before the analysis, serum samples were pretreated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) overnight at 37°C and subsequently heated at 56°C for 45 minute.

Local IgA in nasal swabs was measured by ELISA[18,19] using 16 HAU per 0.05 ml of whole purified viruses for absorption and peroxidase-labeled antibodies to human IgA (BD Biosciences, Franklin Lakes, NJ, USA). The end-point ELISA titers was expressed as the inversed highest dilution that gave an optical density (OD) equal or greater than twice the mean OD of the control (blank) wells. Increases in antibody titers after vaccination by four times or more were considered as antibody conversions.

Local antibody avidity was estimated by ELISA (urea test) as described by de Souza.[20] The method is based on the ability of urea (chaotropic agent) to dissociate antigen–antibody complexes with weak avidity. High-avidity antibodies were detected by ELISA, measuring the difference between the OD values because of antibody binding in the absence and in the presence of 5 m urea. Assay was slightly modified for nasal swabs. Samples were diluted 1:8 with PBS and added to six wells with adsorbed antigen. After 1 hour of incubation, wells were washed with PBS–Tween20. Then, 5 m urea solution was added into three wells, and three extra wells were used as controls (PBS instead of urea). Plates were incubated for 10 minutes at room temperature and washed. Then, antibodies to human IgA were added to the wells (BD Biosciences). After 1 hour of incubation, OPD substrate was added to the wells for 20 minutes, and finally stop solution (2N H_2SO_4) was added. Avidity index (AI) was calculated as the ratio between mean OD of sample treated with urea and the mean OD of controls ×100.

Levels of $CD4^+$ $CD45R0^+$ cells were measured by flow cytometry. Cells were stained with respective monoclonal

antibodies (BD Biosciences) using standard manufacturer's protocols. Analysis was performed using Coulter Epics XL (Beckman Coulter, Inc., Brea, CA, USA).

Virus-specific T cells were detected by the TRAP method based on previous protocols[16,21] Human PBMCs were divided into 'effectors' and 'targets' (Figure 1). Half the targets were loaded with virus by exposure to 1·5 MOI of purified A/17/Solomon Islands/06/9 (H1N1) influenza virus for 1 hour in serum-free RPMI at 37°C in 5% CO_2. The other half of the target cells were stimulated with PBS instead of virus and used as a control. Loaded targets were washed in RPMI–10%FBS and incubated overnight at 37°C in 5% CO_2 followed by labeling of the target cells. Target cells were washed twice with PBS and surface biotinylated using a biotinylation reagent-EZ-Link Sulfo-NHS-LC-Biotin (Merck & Co., Inc., Whitehouse Station, NJ, USA) at a final concentration of 1 mg/ml. Cells were

incubated for 10 minute at 25°C, 10 minute with equal volume of FBS (fetal bovine serum) at 4°C, and washed three times with RPMI–10%FBS. Effector cells were labeled with 0·05 μm CFSE (Sigma-Aldrich Co.), then incubated for 5 minute at room temperature in the dark, and washed three times in PBS–10%FBS. The effector cells were then co-cultured with loaded or control targets in a 1:1 ratio for 1 hour at 37°C in 5% CO_2 to allow the trogocytosis to occur. The cells were then treated with cold PBS–EDTA, washed in PBS, and stained with the phycoerythrin-conjugated monoclonal antibodies to human CD4 (CD4-PE; BD Biosciences) and phycoerythrin–Texas Red streptavidin (second-step reagent for the indirect immunofluorescent staining of biotinylated cells – StAv-PE-TR, (Beckman Coulter, Inc.). The samples were analyzed by flow cytometry using a Coulter Epics Altra (Beckman Coulter, Inc.).
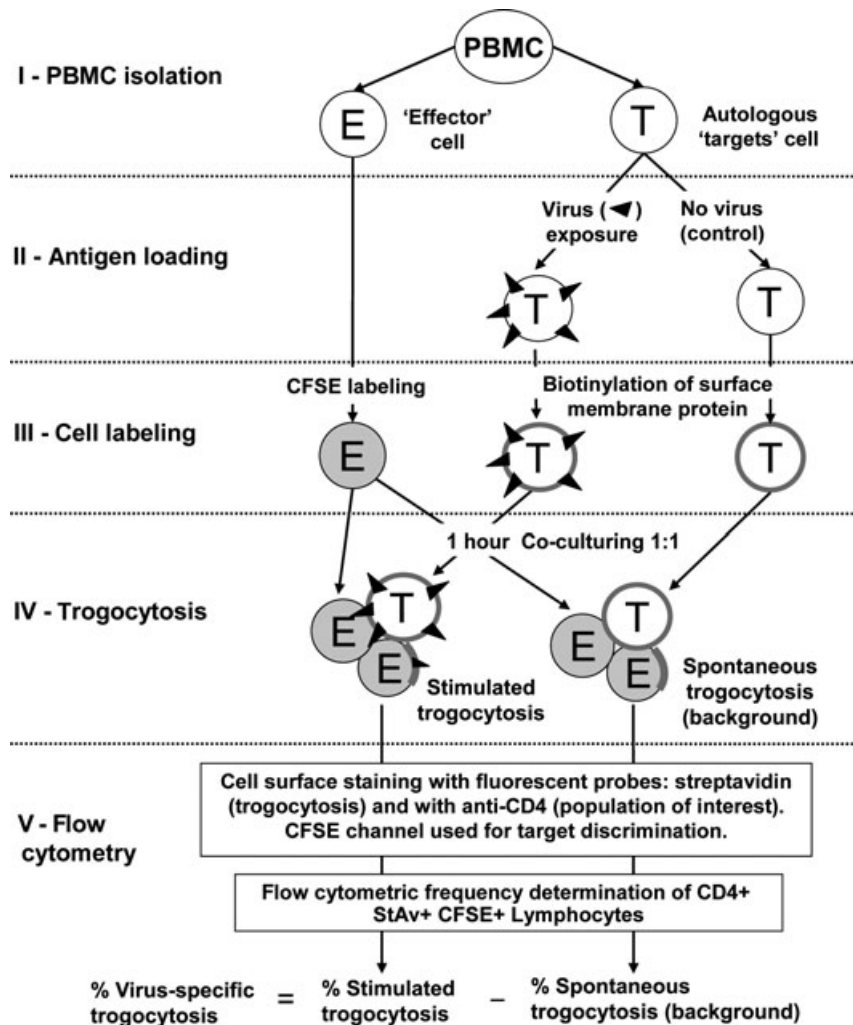

Figure 1. T-cell recognition of antigen-presenting cells assay. PBMC, peripheral blood mononuclear cells; E, effector cells; T, target cells (antigen presenting cells); CFSE, carboxyfluorescein succinimidyl ester; StAv, fluorescently labeled streptavidin.

Statistical analysis was performed using Wilcoxon matched pair test, Mann–Whitney *U*-test and *T*-test. Spearman's coefficient (*r*) was calculated for correlations. *P*-values of <0·05 were considered statistically significant.

Results

Geometric mean titers (GMTs) of local IgA in 56 volunteers immunized with A (H1N1) LAIV strain increased from 68·9 to 164·0 by 21 days after vaccination ($P < 0·01$). Conversion rate (percentage of volunteers showing a fourfold or higher HI antibody titer increase 21 days after vaccination) was 50% in vaccinated persons. Subject received placebo had no conversions with GMT of 46·5 (Figure 2). Thus, A (H1N1) LAIV strain was able to induce marked local antibody response.

Analysis of the local IgA avidity was carried out in the same volunteers divided into three groups: (i) vaccinated people with significant increases in local IgA antibody titers in ELISA; (ii) vaccinated people without such increases; and (iii) placebo group (Table 1). Wide range of avidity indexes (AIs) of local IgA was observed in volunteers before vaccination (day 0): from 33 to 96 units. We divided all volunteers into three subgroups: low AIs (33–60), medium AIs (61–80), and high AIs (81–96). The percentage of volunteers with high baseline AIs among ELISA(+) persons (with fourfold or higher conversions in local IgA at day 21) was 7·9 times lower than among ELISA(−) persons (without local IgA conversions): 3·6% compared to 28·6% ($P < 0·05$). The opposite distribution was observed in volunteers with low baseline AIs, 50·0% of ELISA(+) subjects, and 21·4% of ELISA(−) subjects. After vaccination with LAIV, the percentage of subjects with high AIs among the ELISA(+) volunteers increased 8·9 times: from 3·6% to 32·1%. In ELISA(−) group, the vaccine-induced increase was less:
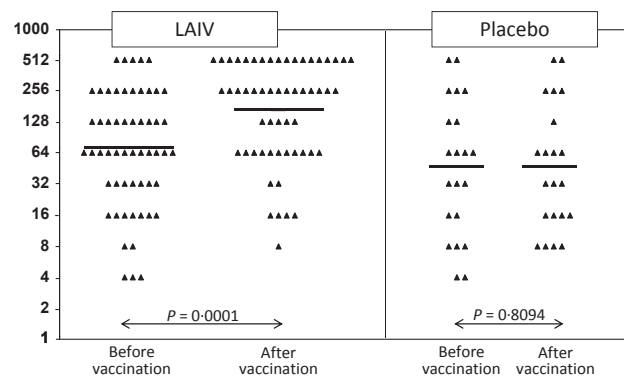

Figure 2. Local IgA titers before and after live attenuated influenza vaccines (LAIV) vaccination. Ab titers were measured before vaccination and 21 days after vaccination in volunteers vaccinated with attenuated reassortant strain for LAIV A/17/Solomon Islands/06/9 (H1N1). Triangles designate individual ELISA titers. Solid bars designate GMTs. GMTs of local IgA increased after LAIV vaccination. No changes were observed in the placebo group. *T*-test for dependent samples was used for *P* calculation.

from 28·6% to 35·7%. During the period between nasal swab sample collections (from day 0 to day 21) some people demonstrated decreased AI results. The percentage of persons with AI decreases was minimal (14·3%) among the ELISA(+) volunteers, medium (32·1%) among the ELISA(−) volunteers, and largest (57·1%) in the control group. We established the cutoff point based on placebo data (increases by 15 and more) to consider the changes in vaccinated subjects to be reliable. It is important to note that such increases were observed not only in ELISA(+), but also in ELISA(−) volunteers (28·6% and 21·4% of persons, respectively). Thus, enhancement of local IgA levels after vaccination was accompanied by increase in their avidity. Furthermore, vaccination delayed decrease in AIs in com-

Table 1. Avidity Indexes (AIs) of local IgA antibodies to A (H1N1) LAIV prior and after vaccination of volunteers

Group	Antibody conversions to A (H1N1) vaccine strain in ELISA: (+): presence (−): absence	*n*	Number and percentage (within bracket) of persons prior vaccination with following AIs			Number and percentage of persons with high AIs (81–96) after vaccination	*N*-fold changes in AIs after vaccination	Number and percentage of persons with AI changes after vaccination	
			AIs from 33 to 60	AIs from 61 to 80	AIs from 81 to 96			AI decreases by 2 or more	Reliable* AI increases
LAIV	ELISA (+)	28	14 (50·0)	13 (46·4)	1 (3·6)	9 (32·1)	8·9	4 (14·3)	8 (28·6)
	ELISA (−)	28	6 (21·4)	14 (50·0)	8 (28·6)	10 (35·7)	1·3	9 (32·1)	6 (21·4)
Placebo	ELISA (−)	21	6 (28·5)	11 (52·4)	4 (19·1)	2 (9·5)	0·5	12 (57·1)	0

*Increases exceeding maximal value observed in the placebo group were considered significant (AI increase by 15 and more) LAIV, live attenuated influenza vaccines.
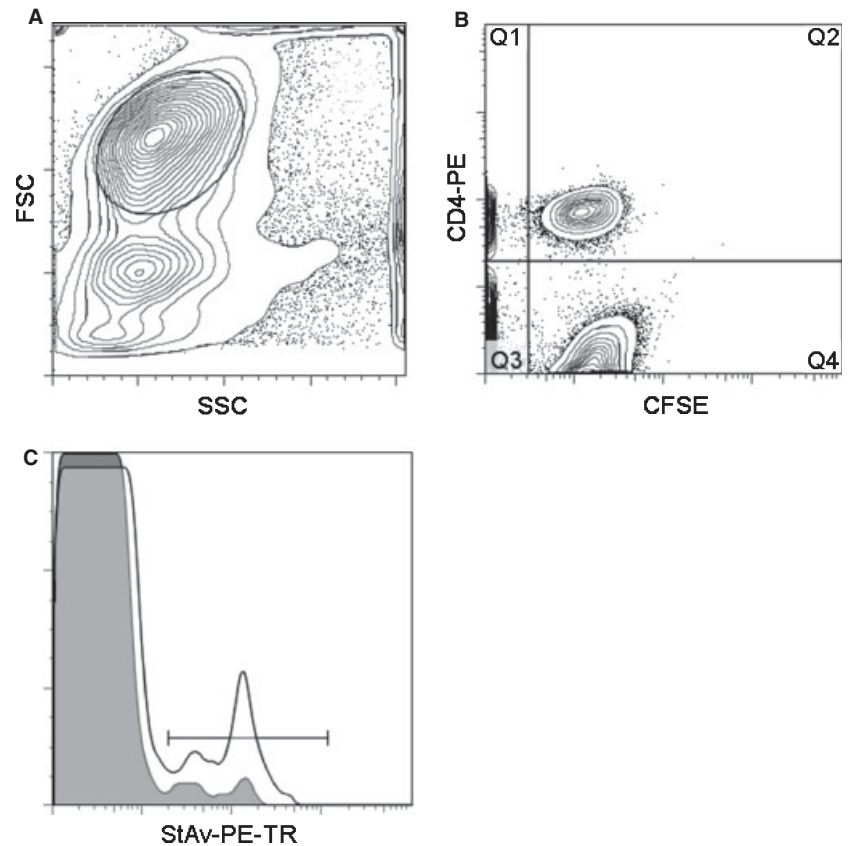
Figure 3. T-cell recognition of antigen-presenting cells analysis by flow cytometry. (A) Lymphocyte gating by light scattering. (B) Determination of CFSE⁺ CD4⁺ T cells (gate Q2). (C) Quantification of CFSE⁺ CD4⁺ T cells involved in trogocytosis (StAv-PE-TR+). White histogram: virus-stimulated sample; grey histogram: control sample (RPMI medium only). Percent of virus-specific cells was calculated as a difference between percents of cells involved in trogocytosis in virus-stimulated and control samples. The gate of trogocytosis-positive cells (C) was stated using negative control (non biotinylated targets stained with anti- CD4 antibodies and streptavidin).

parison with the control group. There is a strong and significant negative correlation between AIs before vaccination and fold changes in AIs after vaccination ($r = -0.63$, $P < 0.05$), meaning that the higher the baseline avidity, the lower the intensity of vaccine-caused changes in Ab avidity. A weak but significant positive correlation was also found between IgA antibody titers, and AI was 0.31 ($P < 0.05$).

The CD4 memory T-cell responses were measured by surface CD45RO staining showing memory phenotype – total memory T cells (in volunteers vaccinated with trivalent LAIV) – and by new TRAP assay adapted to study influenza-specific memory T-cell responses in human PBMC [in persons vaccinated with A (H1N1) reassortant strain] (Figure 4). Examples of lymphocyte gating and quantification of CFSE+ CD4+ T cells are presented in Figure 3. Changes in CD45RO-expressing total memory CD4 cells and trogocytosis-reactive virus-specific CD4 T cells were analyzed in three groups of volunteers: (i) HAI(+) persons – with seroconversions in HAI test, (ii) HAI(−) persons – without seroconversions, and (iii) placebo group. Increases in both total CD4⁺ CD45RO⁺ cells and virus-specific CD4⁺ were more marked in HAI(+) volunteers than in HAI (−). This difference was much more obvious for virus-specific cells. In the control groups, the decline in the levels of these cells was observed within

the 1-month period. The average CD4 T-cell levels before vaccination in HAI(+) group were much lower than in HAI(−) group: 0.014% and 0.170%, respectively ($P < 0.01$). As has been shown in the mucosal IgA avidity results, here we observed a similar decrease in the total and virus-specific CD4 T cells in some volunteers within 1-month period after vaccination. Proportion of such persons among HAI(+) persons was 28.6% (total) and 20.0% (virus specific), among HAI(−) was 43.3% and 40.0%, and in control group 72.0% and 70.0%.

Analysis of the individual data using *n*-fold changes in total CD4⁺ CD45RO⁺ cells and virus-specific CD4⁺ cells in vaccinated volunteers and placebo group (Figure 5) allowed us to establish the following cutoff points: more than 1.5-fold increase for total and more than 10.0-fold increase for virus-specific CD4 memory T cells. Results below cutoff were considered as non-specific. Considering these criteria, the percentages of persons who provided reliable increases in total and influenza-specific CD4 T cells were 42.9% and 70.0% in HAI(+) group and 22.2% and 40.0% in HAI(−) group. Thus, vaccination with LAIV enhanced the levels of total CD4⁺ CD45RO⁺ and virus-specific CD4⁺ memory cells not only in volunteers with seroconversions, but in persons with no seroconversions as well. Furthermore, we found a strong and significant negative correlation between
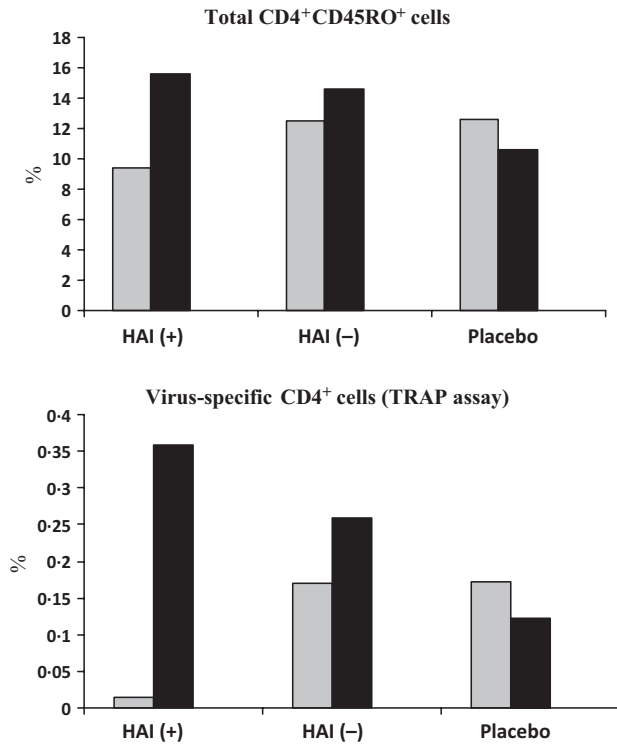
Figure 4. T-cell immunological memory in volunteers after live attenuated influenza vaccines (LAIV) vaccination. Y axis is the arithmetic mean percent of cells. Levels of CD4^{+} CD45RO^{+} cells were measured in volunteers vaccinated with trivalent LAIV [A/17/New Caledonia/99/145 (H1N1), A/17/California/04/6 (H3N2) and B/60/Jilin/01/1]. Virus-specific memory T cells were studied in persons vaccinated with attenuated reassortant strain for LAIV A/17/Solomon Islands/06/9 (H1N1). [Hemagglutination inhibition HAI (+)]: volunteers with serum antibody conversions in HAI test; HAI (−): volunteers without seroconversions. Grey bars: before vaccination, black bars: 1 month after vaccination. Increases were more marked in HAI(+) volunteers, than in HAI ($P < 0.01$). Wilcoxon matched pair test was used for comparison of vaccine and placebo groups, Mann–Whitney *U*-test was used for estimation of changes within groups before and after vaccination.

Figure 5. T-cell immunological memory in volunteers after live attenuated influenza vaccines (LAIV) vaccination. Levels of CD4^{+} CD45RO^{+} cells were measured in volunteers vaccinated with trivalent LAIV [A/17/New Caledonia/99/145 (H1N1), A/17/California/04/6 (H3N2) and B/60/Jilin/01/1]. Virusspecific memory T cells were studied in persons vaccinated with attenuated reassortant strain for LAIV A/17/Solomon Islands/06/9 (H1N1). Triangles designate individual fold-changes (% of cells before vaccination/% of cells 1 month after vaccination). Solid bars designate medians and dotted lines the cutoffs. The following increases were accepted as significant: for total CD4^{+} CD45RO^{+}memory cells, results exceeding maximal value in placebo group in 1.5 times and more; for virus-specific CD4^{+} cells, results exceeding maximal value in placebo group in 10.0 times and more. Mann–Whitney *U*-test was used for *P* calculation.

cell levels before vaccination and fold changes in cell levels after vaccination ($r = -0.48$ with $P < 0.05$ for total CD4 T cells; $r = -0.52$ with $P < 0.05$ for influenza-specific ones).

Discussion

The ability of long-living plasma cells to produce high-avidity antibodies (effector antibodies) is one of the most important components of B-cell memory;[5] therefore, virus-specific B-cell memory can be estimated by the measurement of antibody avidity. Concerning anti-influenza vaccination, local (mucosal) memory B cells are of the greatest interest as mucosal antibodies provide the first immunological barrier against influenza infection.[22] In our study of local IgA antibody avidity, we applied an assay that has
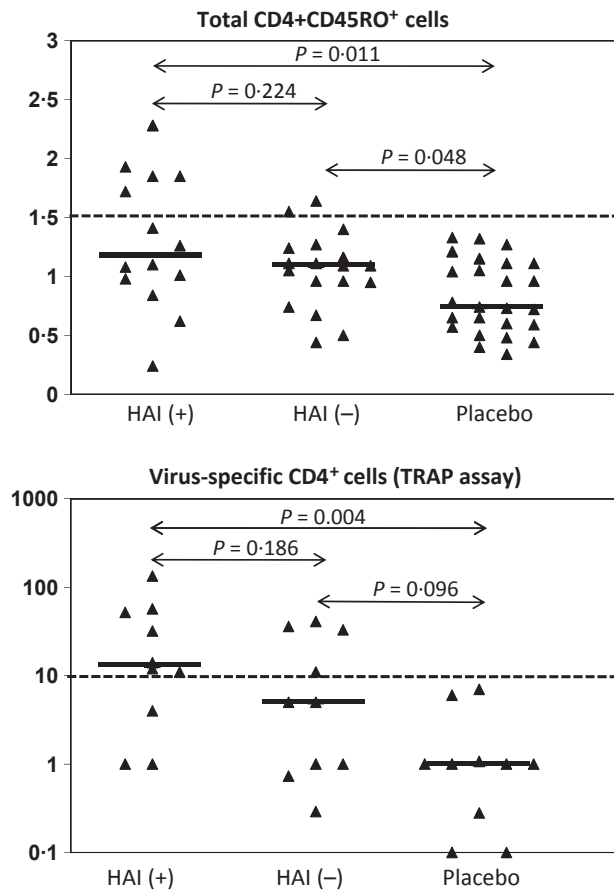
been previously used only for serum antibody avidity evaluation.[18,19,23]

It has been observed that clearance of LAIV influenza strains was completed at day 7–day 10.[24] Following antigen elimination, more than 90% of lymphocytes die by apoptosis with the remaining cells transforming into memory subpopulation.[25] We chose CD4 T cells for memory T-cell assessment as they have been shown to participate in the regulation of both cytotoxic and antibody immunity.[26] Total memory CD4 T cells were determined by the expres-

sion of CD45RO as a general marker of memory pheno-type.[11] Influenza-specific memory CD4 T cells were measured by functional test using the new TRAP assay based on recently described phenomenon of trogocytosis.

The seasonal LAIV vaccine was able to stimulate B-cell memory responses by increasing local IgA avidity in the upper airways, i.e., in the region of the first contact of influenza virus with a host (Table 1). Interestingly, some persons provided reliable increases in IgA avidity without simultaneous reliable increases in mucosal antibody titers. The same phenomenon was previously described for serum antibody avidity in influenza-infected patients and volunteers vaccinated with influenza vaccines.[23] These data show that production of antibodies with high avidity can occur without significant increases in their titers.

Intranasal vaccination with LAIV increased the levels of both total and virus-specific CD4 memory T cells in human peripheral blood (Figures 4 and 5). It has been previously shown that vaccination of young subjects with inactivated influenza vaccine slightly enhanced the total $CD4^+$ $CD45RO^+$ memory T cells, but the proportion of people with such increases was <20%.[11] Stimulation of total memory T cells seems to be associated with the phenomenon of polyclonal activation of heterotypic lymphocyte subpopulations specific to other antigens (bystander activation).[27–29] The LAIV FluMist (MedImmune, Gaithersburg, MD, USA) was shown to be able to stimulate influenza-specific IFNγ-producing CD4 T cells.[12,30] Activation of total and virus-specific memory T cells is an important tool for homo- and hetero-subtype protection against influenza viruses. However, the data of vaccine-induced polyclonal activation of memory T cell specific to allergens or autoantigens are very limited. We consider this question requires further investigation concerning yearly influenza vaccination particularly in immunocompromised populations, although our recent studies showed that LAIV did not stimulate the production of serum and local antibodies to ovalbumin both in mice sensitized to this antigen[31] and in patients with high total IgE levels.

The analysis of antibody avidity, total, and influenza-specific memory CD4 T cells indicated a strong negative correlation between baseline levels before vaccination and the rate of vaccine-induced immune responses (fold changes in AIs or cell levels). Thus, immunogenicity of influenza vaccines depends on immune status before vaccination, which greatly varies in population. Unfortunately, this factor is not taken into account for the massive immunization, which means that significant proportion of vaccine recipients, particularly those with long vaccination history, may not be able to respond appropriately to the actual vaccine. Therefore, we consider the measurement of B- and T-cell memory before vaccination is necessary for selecting volunteers in newly developed vaccine trials or for evaluating the epidemiological potential of influenza A viruses and pandemic LAIV candidates to make a clear assessment of the actual vaccine benefits.

Significant increases in total and virus-specific memory CD4 T-cell levels were observed not only in vaccinated persons with seroconversions in HAI assay, but apparently also in seronegative individuals (22–40% of volunteers, Figures 4 and 5). These results indicate that the HAI assay (currently standard method for LAIV immunogenicity evaluation) does not properly reflect the frequency and quality of the immune responses elicited by LAIV vaccination and should be combined with other assays related to cellular and mucosal immune responses.

Seasonal LAIV is highly effective in stimulating mucosal B-cell memory proved by significant increases in IgA avidity even in persons without conversions of mucosal Ab titers after vaccination. Intranasal immunization with LAIV enhances total and influenza-specific CD4 T-cell levels in peripheral blood. IgA avidity and memory CD4 T-cell responses do not correspond to immunogenicity rate measured by standard HAI assay. Assessment of cellular and mucosal immune responses is highly recommended for LAIV immunogenicity evaluation.

Mucosal IgA avidity assessment and TRAP assay can be used for studies of anti-influenza immunity after infection and vaccination in both volunteers and animal studies. The main advantage of the avidity assay is its ability to estimate memory B-cell function via antibodies secreted by long-lived plasma cells localized in the mucosa without having to harvest lymphoid tissue from the upper airways. TRAP assay is easier to perform than standard cytokine tests[21] and can reveal lymphocyte–APC interaction at an early stage (even at 1 hour). Further comparative studies will be necessary to determine whether the TRAP assay is superior to the intracellular cytokine staining test for analyzing influenza vaccine immunity.

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