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Age-Associated Change in the Frequency of Memory CD4⁺ T Cells Impairs Long Term CD4⁺ T Cell Responses to Influenza Vaccine¹

Insoo Kang,^{2*} Myung Sun Hong,* Helena Nolasco,* Sung Hwan Park,^{3*} Jin Myung Dan,* Jin-Young Choi,* and Joe Craft*[†]

We investigated the relationship of memory CD4⁺ T cells with the evolution of influenza virus-specific CD4⁺ T cell responses in healthy young and elderly people. Elderly individuals had a similar frequency of CD69⁺CD4⁺ T cells producing IFN- γ and TNF- α at 1 wk, but a lower frequency of these CD4⁺ T cells at 3 mo after influenza vaccination. Although the elderly had a higher frequency of central memory (CM; CCR7⁺CD45RA⁻) CD4⁺ T cells, they had a significantly lower frequency of effector memory (EM; CCR7⁻CD45RA⁻) CD4⁺ T cells, and the frequency of the latter memory CD4⁺ T cells positively correlated with the frequency of influenza virus-specific CD69⁺CD4⁺ T cells producing IFN- γ at 3 mo. These findings indicate that the elderly have an altered balance of memory CD4⁺ T cells, which potentially affects long term CD4⁺ T cell responses to the influenza vaccine. Compared with the young, the elderly had decreased serum IL-7 levels that positively correlated with the frequency of EM cells, which suggests a relation between IL-7 and decreased EM cells. Thus, although the healthy elderly mount a level of CD4⁺ T cell responses after vaccination comparable to that observed in younger individuals, they fail to maintain or expand these responses. This failure probably stems from the alteration in the frequency of CM and EM CD4⁺ T cells in the elderly that is related to alteration in IL-7 levels. These findings raise an important clinical question about whether the vaccination strategy in the elderly should be modified to improve cellular immune responses. *The Journal of Immunology*, 2004, 173: 673–681.

Age-associated changes occur in T cell subsets and function in normal humans (1). The healthy elderly have an increased proportion of memory CD4⁺ and CD8⁺ T cells expressing the surface marker CD45RO and a decreased proportion of naive T cells expressing CD45RA (2–4), although the absolute and relative numbers of CD4⁺ and CD8⁺ T cells appear to remain unchanged with aging (1). Elderly individuals have decreased T cell proliferative responses to mitogens in vitro and less IL-2 secretion from Ag-stimulated T cells compared with young people (1, 5, 6), perhaps related to decreased expression of CD28, a costimulatory molecule critical for T cell activation (7, 8). Studies of T cell cytokine synthesis in the elderly have yielded conflicting data. Unchanged, decreased, and increased production of IFN- γ , a Th1-type cytokine, have been reported from studies where PBMCs were stimulated with mitogens (9–11), although cells other than T cells in PBMCs produce this cytokine.

Two subsets of memory T cells with different effector functions and homing capacities can be discerned in peripheral blood of humans based upon expression of CCR7, a lymph node-homing chemokine receptor (12–14). CD45RA⁻CD4⁺ memory T lymphocytes expressing CCR7 are considered central memory (CM)⁴ cells that have the ability to home to lymphoid organs based in part on their CCR7 expression. Functionally, these cells stimulate dendritic cells efficiently and secrete IL-2. By contrast, CD45RA⁺CD4⁺ memory T lymphocytes that lack CCR7 expression are effector memory (EM) cells that travel to nonlymphoid tissues. These cells have strong proliferative capacity and secrete the effector cytokines, IFN- γ , IL-4, and IL-5. Given the functional difference in these two populations, it is possible that altered T cell memory capacity in the elderly can be secondary to changes in the numbers and/or function of these two subsets. In this study we addressed this possibility by measuring the frequency of CM and EM CD4⁺ T cells in healthy young and elderly people.

Although T cell subsets and function appear to change with aging, the effects of these age-associated changes on the development of in vivo responses to pathogenic Ags are largely unknown. Knowing the answer to this question is critical, because the elderly are more susceptible to infections, with increased morbidity and mortality (15), and CD4⁺ T cells are essential in defending hosts against microbial infections (16). In this study we addressed this question and its possible mechanism by measuring CD4⁺ T cell immune responses to influenza vaccine and correlating such responses with different subsets of CD4⁺ T cells in healthy young and elderly people. We have chosen to use influenza virus to measure CD4⁺ T cell immune responses, because infection with this virus is a serious public health problem causing 20,000–40,000 deaths and up to 300,000 hospitalizations each year in the U.S.

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⁴ Abbreviations used in this paper: CM, central memory; EM, effector memory.

(17), with the greatest morbidity and mortality from this infection occurring in people aged 65 years and older (18). These findings suggest that age-related changes in immunity contribute to an impaired immune response to influenza virus. Indeed, elderly individuals have decreased Ab responses to influenza virus infection and influenza vaccination (15) as well as decreased CD8⁺ T cell cytotoxicity against influenza virus (19).

In the current study we prospectively determined CD4⁺ T cell responses specific for individual strains of virus in the influenza vaccine in healthy young and elderly people before and at 1 wk and 3 mo after vaccination, using a whole blood, short term, Ag stimulation assay (20). In addition, we measured the frequencies of naive, CM, and EM CD4⁺ T cells and correlated these findings with CD4⁺ T cell responses to influenza vaccine. Recently, studies reported a role for IL-7 in sustaining the survival of memory CD4⁺ T cells (21–23), suggesting that any change in the number and function of memory CD4⁺ T cells in the elderly could be secondary to alterations in the serum IL-7 level and IL-7R α expression. Therefore, we measured serum IL-7 levels and IL-7R α expression on CD4⁺ T cells in the young and the elderly. We found that the elderly mounted influenza virus-specific CD4⁺ T cell responses as effectively as did the young at 1 wk after vaccination, but had decreased influenza virus-specific CD4⁺ T cell responses at 3 mo. We also noted a decreased level of serum IL-7, an increased frequency of CM CD4⁺ T cells, and a decreased frequency of EM CD4⁺ T cells in the elderly. We found a correlation of the frequency of EM CD4⁺ T cells with serum IL-7 levels and influenza virus-specific CD4⁺ T cell responses at 3 mo after vaccination. However, IL-7R α expression by CD4⁺ T cells was not different between the young and the elderly. These findings suggest that elderly individuals have impaired long term CD4⁺ T cell immune responses to the influenza vaccine that probably stem from the altered balance in memory CD4⁺ T cell subsets, as evidenced by the change in the frequency of CM and EM CD4⁺ T cells that is related to IL-7 levels.

Materials and Methods

Human subjects

Healthy elderly subjects, 65 years of age ($n = 13$) or older, and healthy young subjects, 40 years of age or younger ($n = 10$), were recruited for this study. The mean ages \pm SD for young and elderly subjects were 33.9 ± 5.7 and 75.7 ± 5.8 years, respectively. The gender distribution was not significantly different between the two groups ($p = 0.102$, by Fisher's exact test). Individuals who were taking immunosuppressive drugs or who had a disease potentially affecting the immune system, including cancer, autoimmunity, and diabetes were excluded. All subjects were vaccinated in October and November of 2002 with a commercially available subvirion trivalent 2002–2003 influenza vaccine (FluShield; Wyeth Laboratory, Madison, N.J.) containing the following strains: A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Hong Kong/330/2201. Peripheral blood was collected in heparinized tubes before vaccination and 1 wk and 3 mo later. None of the young and elderly subjects had a serious infection requiring hospitalization during the study period. In addition, nine healthy elderly people and nine healthy young people were recruited for evaluation of naive, CM, and EM CD4⁺ T cells. The mean ages \pm SD for young and elderly subjects were 33.6 ± 4.2 and 72.7 ± 4.3 years, respectively. The gender distribution was not significantly different between the two groups ($p = 0.667$, by Fisher's exact test).

Informed consent was obtained from all subjects. This work was approved by the institutional review committees of Yale University and the Veterans Administration New England Health Care System, West Haven Campus.

Analysis of influenza virus-specific CD69⁺CD4⁺ T cells by multiparameter flow cytometry after short term in vitro Ag stimulation

Heparinized blood was aliquoted in 15-ml conical polypropylene tubes (BD Biosciences, Franklin Lakes, NJ) at 1 ml/tube, as previously described

(20). The costimulatory anti-CD28 and -CD49d mAbs (BD Pharmingen (San Diego, CA) and BD Immunocytometry Systems (San Jose, CA), respectively) were added to the samples at 1 μ g/ml, respectively, with the following whole viral proteins at optimal stimulatory concentrations: influenza virus A/New Caledonia, A/Panama, and B/Hong Kong vaccine concentrate (provided by Dr. R. Hjorth, Wyeth Laboratory) and influenza virus matrix protein (from Dr. D. Bucher, Albany Medical College, Albany, NY). Lysates of uninfected cells (Microbix, Toronto, Canada) were added as a negative control for viral Ags. Culture tubes were incubated in a humidified atmosphere at 37°C in 5% CO₂ incubator for 6 h, with the last 4 h of incubation in the presence of brefeldin A (10 μ g/ml; Sigma-Aldrich, St. Louis, MO), a secretion inhibitor, to enhance the accumulation of cytokines in the cytoplasm. At 6 h, 100 μ l of 20 mM EDTA, for a final concentration of 2 mM EDTA, was added. RBCs in blood samples were then lysed, and remaining cells were fixed with FACS Lysing Solution (BD Biosciences, San Jose, CA). Cells were subsequently resuspended in BD FACS Permeabilization Solution (BD Biosciences) and stained with Abs to CD4-allophycocyanin, CD69-PE, and IFN- γ or TNF- α -FITC for analysis of influenza virus-specific CD4⁺ T cells.

Data were acquired on a FACSCalibur system (BD Biosciences) and analyzed using Flow Jo software (Tree Star, Ashland, OR). The frequency of CD69⁺CD4⁺ T cells producing cytokines in the negative control stimulated with uninfected cell lysates (Microbix Biosystems, Toronto, Canada) was subtracted from the frequency of the same cell population in a sample stimulated with influenza virus as previously described (24–26). These values obtained after subtracting the negative control were used for the statistical analyses.

Determination of the frequency of naive and central and EM CD4⁺ T cells.

PBMCs were purified from six elderly and six young subjects participating in the influenza vaccine study and from separate groups of nine elderly and nine young people by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) gradient centrifugation of heparinized blood. Cells were washed with wash buffer containing 0.5% BSA and incubated with anti-CCR7 mAb (BD Pharmingen) at 4°C for 20 min. Cells were washed twice and stained with biotinylated rat anti-mouse IgM (BD Pharmingen) at 4°C for 20 min. Cells were again washed twice and incubated with streptavidin-PE, anti-CD4-allophycocyanin, and anti-CD45RA-FITC at 4°C for 45 min, followed by repeat washing and fixation with 2% formaldehyde.

Measuring serum IL-7 levels and expression of IL-7R α in CD4⁺ T cells

Serum was separated from the peripheral blood and stored at -80°C until the analysis. Serum IL-7 levels were measured in 12 young and 12 elderly individuals using a commercially available human IL-7 ELISA kit (Diaclone Research, Canton, MA) according to the manufacturer's instructions. For measurement of IL-7R α expression on CD4⁺ T cells in the young and the elderly, PBMCs from 10 young and nine elderly subjects were washed and incubated with human IgG (1 μ g/10⁵ cells; R&D Systems, Minneapolis, MN) for 15 min for IgG FcR blocking. The Fc-blocked cells were stained with anti-IL-7R α Abs conjugated with PE (R&D Systems) or isotype Abs for 30 min at 4°C. Cells were washed with wash buffer, fixed with 2% formaldehyde, and analyzed as described above.

Determination of anti-influenza virus Abs in serum

Anti-influenza virus Abs in serum obtained from seven young and 11 elderly individuals before and 3 mo after vaccination were measured by ELISA as previously described (27), with some modifications. Briefly, 96-well microtiter plates (ICN Biomedicals, Aurora, OH) were coated overnight at 4°C with lysates of influenza virus (A/New Caledonia, A/Panama, and B/Hong Kong) in coating buffer at 1 μ g/ml. After blocking with 1% BSA, plates were loaded with a 1/6000 dilution of serum in 0.1% BSA, followed by incubation for 2 h at room temperature. This dilution was selected based on the finding of a pilot study using 2-fold serial dilutions (data not shown). Plates were washed and incubated for 1 h at room temperature with goat anti-human IgG Abs conjugated with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). After washing, plates were developed by adding *p*-nitrophenyl phosphate (Sigma-Aldrich). The OD was read at 405 nm. The OD values of individual samples were compared against the OD value of the same internal control serum through the experiments.

Statistical analysis

The IL-7 levels, the OD values of anti-influenza virus Abs, the mean fluorescent intensity of IL-7R α expression, and the mean frequencies of influenza virus-specific CD69⁺CD4⁺ T cells and naive, CM, and EM CD4⁺ T cells in elderly and young subjects were compared by the Mann-Whitney *U* test. The χ^2 test or Fisher's exact test was used to compare a difference in the frequency of vaccine responders and vaccine nonresponders between elderly and young subjects as appropriate. The Pearson correlation was performed to determine bivariate correlations. All statistical analyses were performed using SPSS 10.1 (SPSS, Chicago, IL).

Results

Determination of influenza virus-specific CD4⁺ T cell responses in whole blood after short term *in vitro* stimulation

Influenza virus-specific CD69⁺CD4⁺ T cells producing IFN- γ and TNF- α in whole blood were first identified in healthy young and elderly people before influenza vaccination (Fig. 1A; see representative examples). The mean frequencies of influenza virus-specific CD69⁺CD4⁺ T cells producing IFN- γ and TNF- α were low, ranging from 0.03–0.1% at baseline. Elderly and young people had a similar frequency of IFN- γ - and TNF- α -producing CD69⁺CD4⁺ T cells specific for individual strains of influenza virus and influenza virus matrix protein at baseline ($p > 0.05$; data not shown).

Evolution of influenza virus-specific CD69⁺CD4⁺ T cell responses after influenza vaccination

One week after vaccination, a rise in the frequency of influenza virus-specific CD69⁺CD4⁺ T cells producing IFN- γ and TNF- α typically occurred in individuals (Fig. 1B; see representative examples). Similar responses were detected 3 mo after vaccination (Fig. 1C). The mean increase in the frequency (percentage) of CD69⁺CD4⁺ T cells producing IFN- γ and TNF- α specific for individual strains and for influenza virus matrix protein was similar between the young and the elderly 1 wk after vaccination (mean increase, 0.03–0.08 and 0.03–0.1%, respectively; $p > 0.05$; Fig. 2, A and B). By contrast, 3 mo after vaccination, young people maintained or expanded their CD69⁺CD4⁺ T cells specific for the influenza virus matrix protein as well as for the New Caledonia and Panama strains compared with the elderly who did not ($p < 0.05$; Fig. 2, C and D). A similar trend was noticed in the frequency of CD69⁺CD4⁺ T cells specific for the Hong Kong strain, without reaching statistical significance (Fig. 2, C and D).

Based on the increase in influenza virus-specific CD69⁺CD4⁺ T cells responses after vaccination, subjects were divided into two groups: vaccine responders and vaccine nonresponders. Responders were those who had at least a 2-fold increase in the frequency of influenza virus-specific CD69⁺CD4⁺ T cells producing cytokines

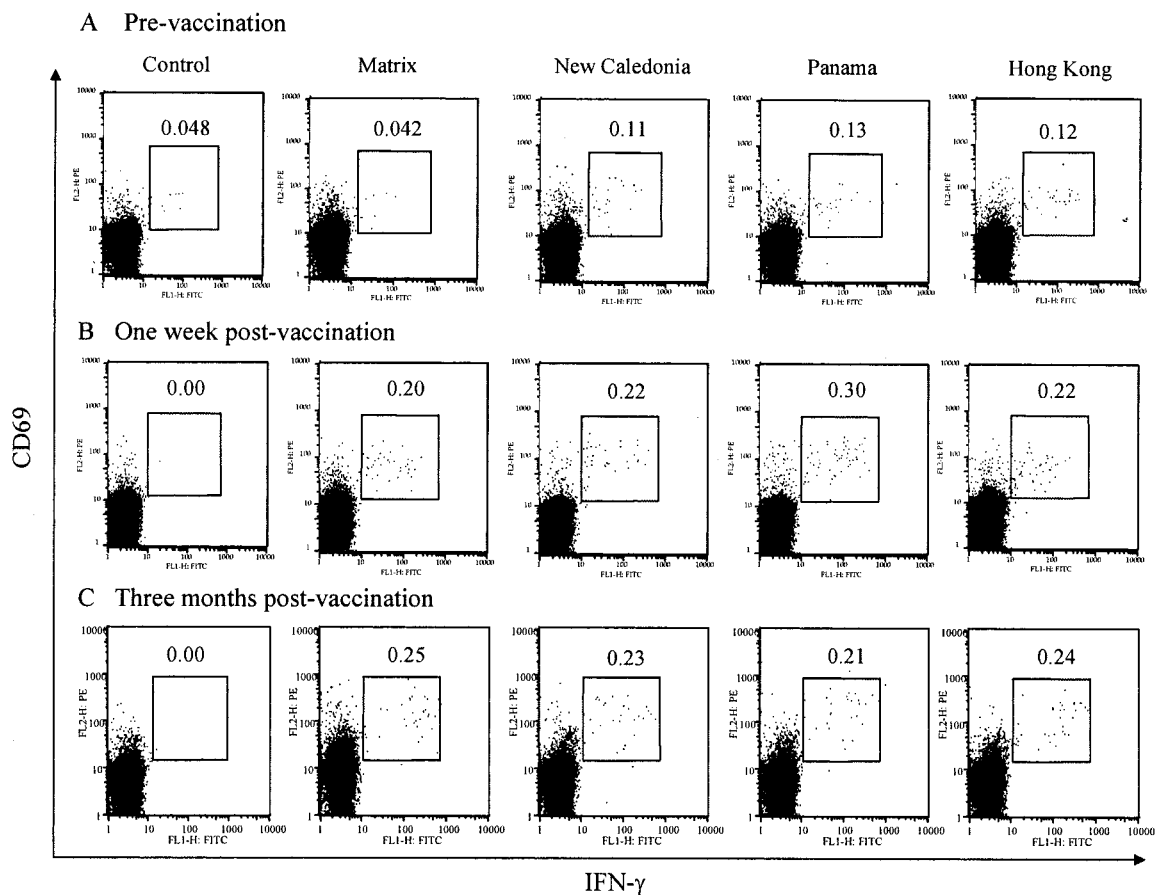


FIGURE 1. Influenza virus-specific CD4⁺ T cells are identified in human peripheral blood before and after influenza vaccination. Peripheral blood was obtained from a healthy individual before (A) and 1 wk (B) and 3 mo (C) after influenza vaccination. Heparinized whole blood was cultured with various stimuli (control Ag, influenza virus matrix protein (matrix), New Caledonia strain, Panama strain, and Hong Kong strain with anti-CD28 and anti-CD49d Abs) and stained with Abs to CD4, CD69, and IFN- γ . Cells were gated on the CD4⁺ population. Specific cytokine expression was measured within the CD69⁺ (activated) cell subset to enhance the identification of Ag-specific CD4⁺ T cells. The percentage of CD69⁺CD4⁺ T cells staining with FITC-conjugated anti-IFN- γ Abs is indicated above the rectangular region of each plot.

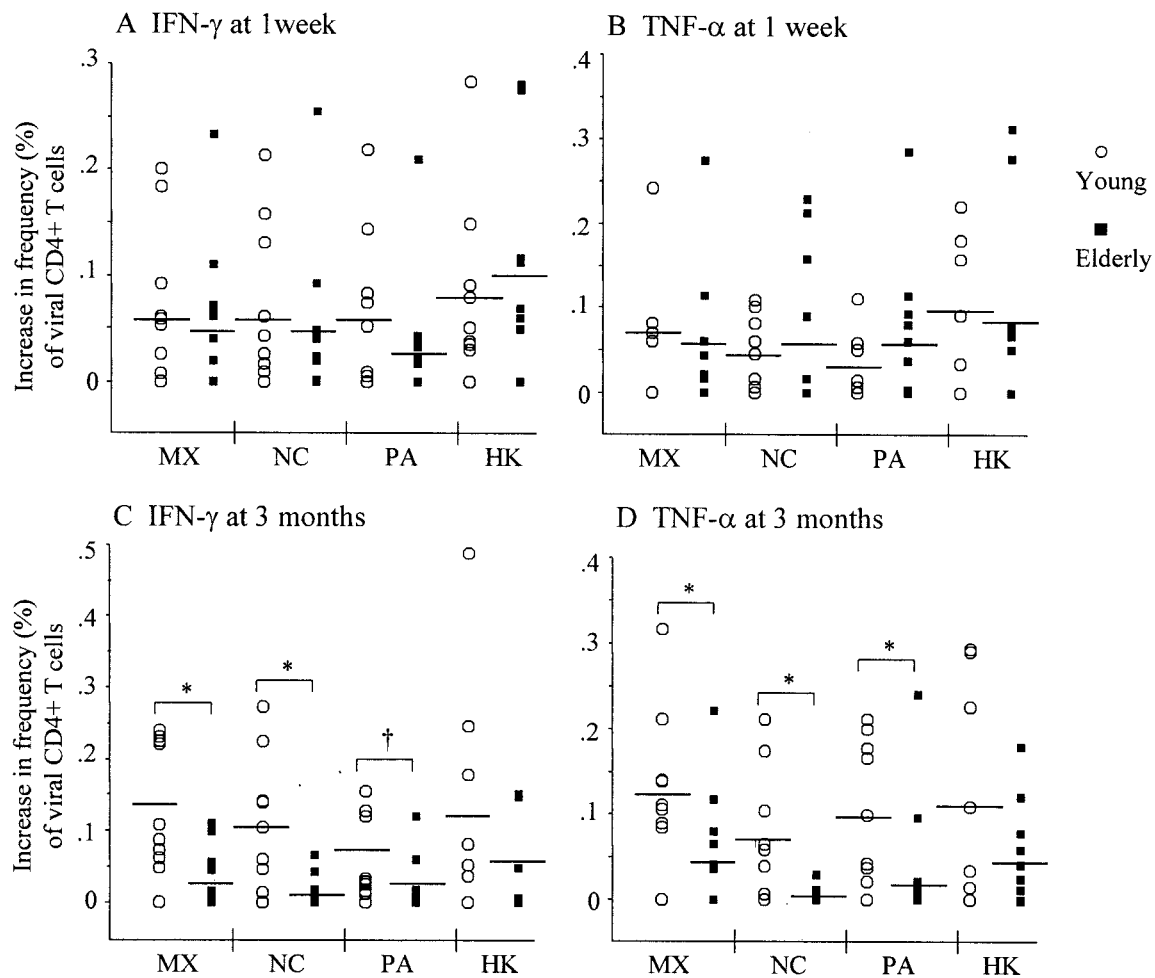


FIGURE 2. Increase in the frequency (percentage) of CD69⁺CD4⁺ T cells specific for influenza matrix protein and three strains of influenza virus in young ($n = 10$) and elderly ($n = 13$) subjects at 1 wk (A and B) and 3 mo (C and D) after influenza vaccination. Horizontal bars indicate the mean increase in the frequency (percentage) of CD69⁺CD4⁺ T cells producing IFN- γ (A and C) and TNF- α (B and D) that are specific for influenza matrix protein (MX) and three strains of influenza virus (New Caledonia (NC), Panama (PA), and Hong Kong (HK)) compared with the baseline (before vaccination). Values on the y-axis are the increase in the frequency (percentage) of CD69⁺CD4⁺ T cells producing the cytokines specific for influenza matrix protein or each strain of the virus 1 wk and 3 mo after vaccination compared with the baseline. The p values were determined using the Mann-Whitney U test. *, $p < 0.05$; †, $p = 0.059$.

from the baseline after influenza vaccination. This definition was used previously by others for the evaluation of influenza virus-specific T cell responses in the elderly after vaccination (28). The number of responders was similar in young and elderly people 1 wk after vaccination, ranging from 40–70% and from 23–53%, respectively ($p > 0.05$; Fig. 3). By contrast, at 3 mo the number of responders to the New Caledonia and Panama strains (IFN- γ - and TNF- α -producing CD4⁺ cells) and the influenza virus matrix protein (IFN- γ -producing CD4⁺ cells only) was significantly lower in elderly than in young subjects (Fig. 3). The number of responders to the Hong Kong strain and that to the influenza virus matrix protein (TNF- α -producing CD4⁺ T cells) was not different between the young and the elderly ($p > 0.05$; Fig. 3).

Determination of the frequency of naive, CM, and EM CD4⁺ T cells in peripheral blood

Three subsets of CD4⁺ T cells can be identified in the peripheral blood of young and elderly individuals based on the expression of CCR7 and CD45RA (12) (Fig. 4, A and B; see representative examples). Similar to published data (2–4), the elderly ($n = 15$) had a decreased frequency of naive CD4⁺ T cells expressing CD45RA compared with the young ($n = 15$; $p = 0.001$; Fig. 4C). In the

CD45RA⁻ memory CD4⁺ T cell population, the frequency of CD4⁺ T cells expressing CCR7 (CM cells) was higher in the elderly than in the young ($p = 0.000$; Fig. 4D). By contrast, the frequency of EM CD4⁺ T cells (CD45RA⁻ CCR7⁻) was lower in the elderly ($p = 0.000$; Fig. 4E).

A similar result was noticed in analysis of peripheral blood from a subgroup of the young ($n = 6$) and the elderly ($n = 6$) who received the influenza vaccine. In these two groups, the mean frequencies \pm SEM of naive, CM, and EM CD4⁺ T cells, comparing the young vs the elderly, were 41.4 ± 1.66 and $21.0 \pm 5.2\%$ ($p = 0.009$), 41.7 ± 2.32 and $67.8 \pm 5.69\%$ ($p = 0.002$), and 13.4 ± 1.07 and $9.97 \pm 0.48\%$ ($p = 0.015$), respectively.

Correlation of the frequency of naive, CM, and EM CD4⁺ T cells with influenza virus-specific CD4⁺ T cell responses

We next correlated the frequency of naive, CM, and EM CD4⁺ T cells with the frequency of CD69⁺CD4⁺ T cells producing IFN- γ and TNF- α specific for the individual strains of influenza virus and influenza virus matrix protein in 12 young and elderly people who received the influenza vaccine. At baseline and 1 wk after influenza vaccination, there was no specific correlation noticed between these variables (data not shown).

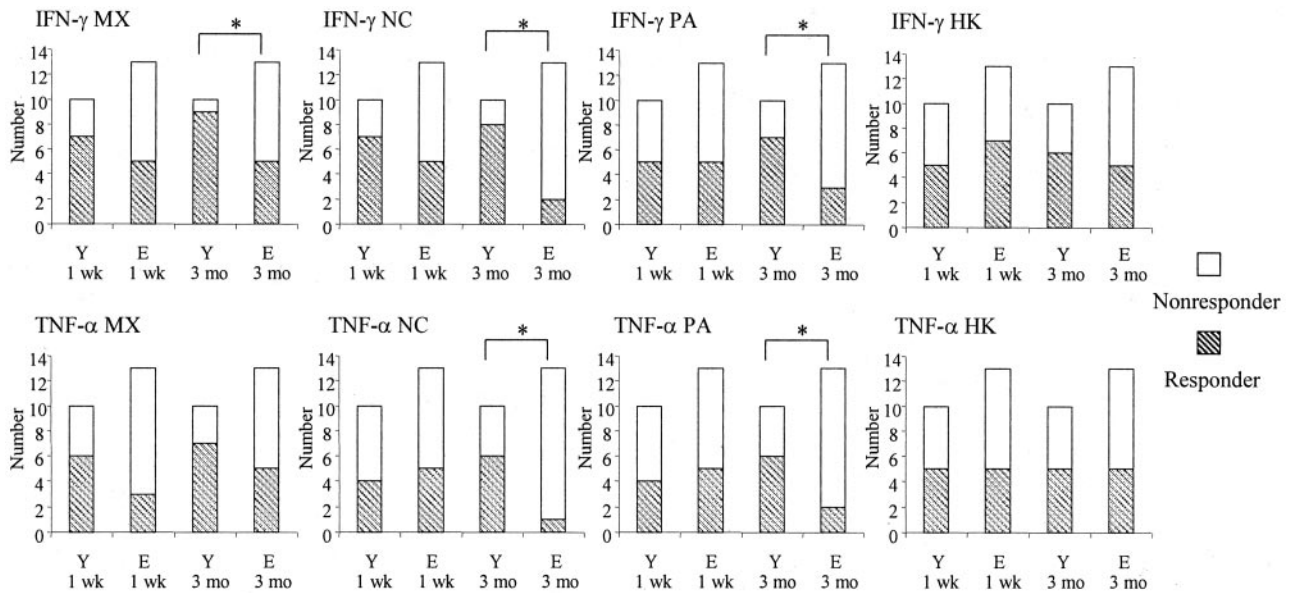


FIGURE 3. Frequencies of CD4⁺ T cell responders and nonresponders to influenza virus matrix protein and influenza virus (New Caledonia (NC), Panama (PA), and Hong Kong (HK)) in young (Y; $n = 10$) and elderly (E; $n = 10$) subjects at 1 wk and 3 mo after influenza vaccination. Responders are defined with a 2-fold or higher increase in the frequency of CD69⁺CD4⁺ T cells secreting IFN- γ and TNF- α that are specific for influenza virus matrix protein and the three strains of influenza virus after vaccination. Values on the y-axis are the number of subjects. The p values were determined using the χ^2 test or Fisher's exact test as appropriate. *, $p < 0.05$.

By contrast, at 3 mo after vaccination, a positive correlation was noticed between the frequency of EM CD4⁺ T cells and the frequency of CD69⁺CD4⁺ T cells producing IFN- γ specific for the influenza virus matrix protein and the New Caledonia and Hong Kong strains (Fig. 5, A, B, and D). A similar trend was found between the frequency of EM CD4⁺ T cells and the frequency of CD69⁺CD4⁺ T cells producing TNF- α specific for the influenza virus matrix protein and the New Caledonia and Hong Kong strains, although a statistically significant correlation was noticed only for New Caledonia-specific CD4⁺ T cells producing TNF- α (data not shown). There was no correlation found between the frequency of influenza virus-specific CD4⁺ T cells producing IFN- γ and TNF- α and the frequency of naive and CM CD4⁺ T cells at 3 mo after vaccination. This finding is not surprising, because naive and CM CD4⁺ T cells are not efficient in producing IFN- γ (12).

Determination of serum IL-7 levels and IL-7R α expression in CD4⁺ T cells

Serum IL-7 levels were measured in 12 healthy young and the same number of healthy elderly people. The elderly had significantly lower levels of serum IL-7 compared with the young ($p = 0.001$; Fig. 6). We correlated the serum IL-7 levels with the frequency of naive, CM, and EM CD4⁺ T cells in the 11 young and elderly individuals. This analysis showed a positive correlation between IL-7 levels and the frequency of naive and EM CD4⁺ T cells ($r = 0.670$; $p = 0.024$ and $r = 0.597$; $p = 0.050$, respectively) and a negative correlation between this cytokine level and the frequency of CM CD4⁺ T cells ($r = -0.688$; $p = 0.019$).

IL-7R α expression on CD4⁺ T cells was next measured in 10 healthy young and nine elderly individuals. Although the expression of IL-7R α was identified in both the young and the elderly, the mean fluorescent intensity of its expression on CD4⁺ T cells was not different between the two groups (mean \pm SEM, 232.5 \pm 20.3 and 228.0 \pm 12.1, respectively; $p = 0.870$).

Measurement of anti-influenza virus Abs in serum

Anti-influenza virus IgG Abs were measured in seven young and 11 elderly individuals. The increase in IgG Abs against New Caledonia, Panama, and Hong Kong strains from the baseline at 3 mo after vaccination was similar between the young and the elderly (mean OD value \pm SEM, 0.264 \pm 0.126 and 0.155 \pm 0.046, 0.258 \pm 0.129 and 0.161 \pm 0.054, and 0.285 \pm 0.185 and 0.467 \pm 0.145, respectively; $p > 0.05$). Although strong correlations were observed among Abs against the three strains of influenza virus in each individuals (data not shown), there was no significant correlation found between anti-influenza virus IgG Ab responses and CD4⁺ T cell responses to this virus ($p > 0.05$).

Discussion

In this study we determined the frequencies of influenza virus-specific CD69⁺CD4⁺ T cells that secreted IFN- γ and TNF- α in the whole blood of young and elderly subjects before and after influenza vaccination. Both groups had a comparable frequency of such cells at 1 wk after vaccination; however, their frequency was significantly lower in the elderly at 3 mo after vaccination (Fig. 2). The number of vaccine responders who had at least a 2-fold increase in the frequency of influenza virus-specific CD4⁺ T cells after vaccination was also significantly lower in the elderly at this later time (Fig. 3). These findings indicate that the elderly mount influenza virus-specific CD4⁺ T cell responses as effectively as the young during the early days following vaccination; however, the elderly cannot expand or maintain these T cell responses as well at later time points.

Of interest, elderly individuals had a decreased frequency of EM (CCR7⁻CD45RA⁻) CD4⁺ T cells and an increased frequency of CM (CCR7⁺CD45RA⁻) CD4⁺ T cells compared with the young. These data indicate that elderly subjects have an alteration in the balance of memory CD4⁺ T cell subsets. As EM CD4⁺ T cells are the primary source of IFN- γ (12), the altered frequency of EM

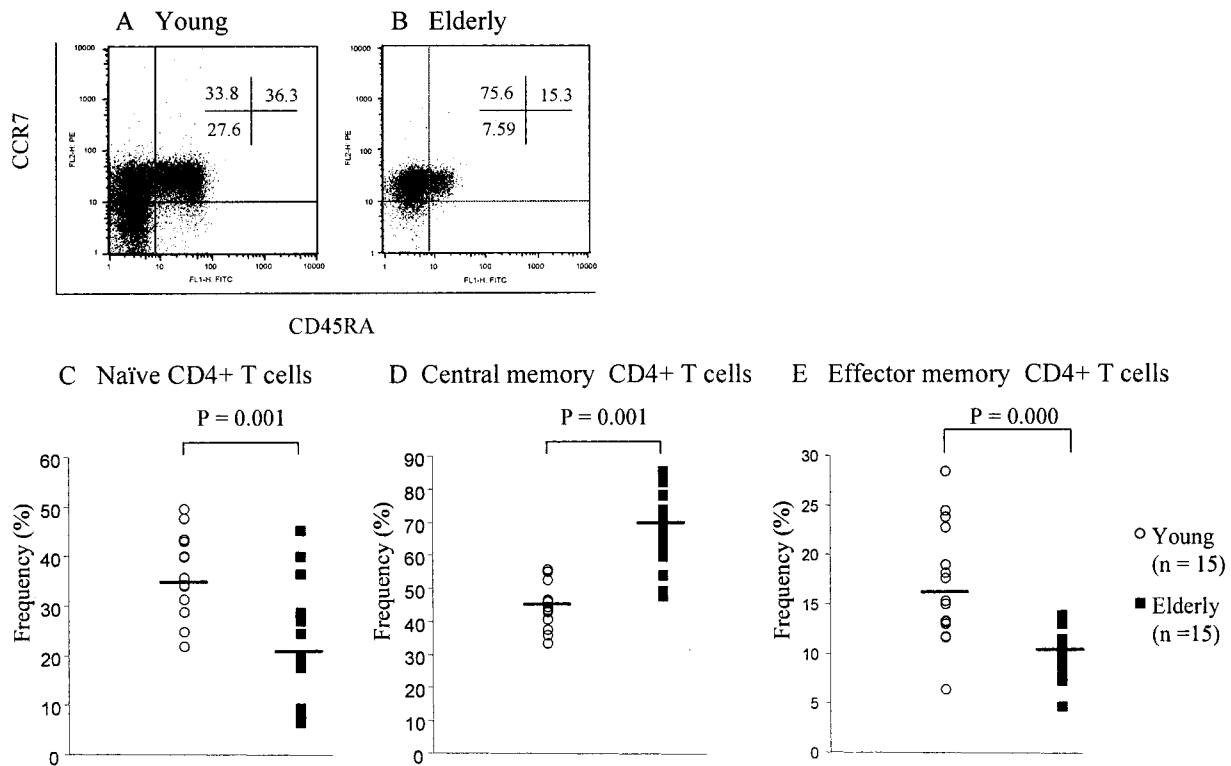


FIGURE 4. Naïve (CD45RA⁺CCR7⁺), central (CD45RA⁻CCR7⁺), and effector (CD45RA⁻CCR7⁻) CD4⁺ T cells in young and elderly subjects. PBMC from 15 young and 15 elderly people were stained with anti-CD4, -CD45RA, and -CCR7 Abs. Naïve cells are CD45RA⁺CCR7⁺. CM and EM cells are CD45RA⁻CCR7⁺ and CD45RA⁻CCR7⁻, respectively. Cells were gated on the CD4⁺ population. Plots *A* and *B* are representative dot plots. The numbers on the plots indicate the percentage of cells in each quadrant (*A* and *B*). Values on the y-axis are the frequency (percentage) of CD4⁺ T cells (*C–E*). Horizontal bars indicate the mean frequency of naïve, CM, and EM CD4⁺ T cells (*C–E*). The *p* were determined using the Mann-Whitney *U* test (*C* and *D*).

CD4⁺ T cells in the elderly is probably responsible for the decreased frequency of virus-specific CD69⁺CD4⁺ T cells producing IFN- γ and TNF- α at 3 mo after influenza vaccination. This idea is supported by data from our study that showed a positive correlation between the frequency of EM CD4⁺ T cells and the frequency of influenza virus-specific CD69⁺CD4⁺ T cells that secreted IFN- γ (Fig. 5). By contrast, there was no correlation found between the frequency of naïve, CM, and EM CD4⁺ T cells and influenza virus-specific CD4⁺ T cell responses at baseline and 1 wk after vaccination. Previous work has shown that EM T cells develop from CM T cells after Ag stimulation (12). Therefore, the absence of correlation between CD4⁺ T cell phenotypes and influenza virus-specific T cell responses at 1 wk after vaccination is not surprising, because influenza virus-specific CD4⁺ T cells producing IFN- γ and TNF- α at this time point are likely to be mixed populations of pre-existing EM CD4⁺ T cells, as noticed at baseline, and new EM CD4⁺ T cells developed from naïve and/or CM CD4⁺ T cells. By contrast, influenza virus-specific CD4⁺ T cells producing IFN- γ and TNF- α at 3 mo after vaccination are probably EM CD4⁺ T cells that have existed for 3 mo or longer and reflect the homeostatic state of EM CD4⁺ T cells. Overall, our data suggest that individuals with a higher frequency of EM CD4⁺ T cells are likely to have a higher frequency of Ag-specific CD4⁺ T cells producing IFN- γ several months after Ag stimulation. This idea provides the explanation for our finding that the elderly with the decreased frequency of EM CD4⁺ T cells also have decreased influenza virus-specific CD4⁺ T cell responses 3 mo after vaccination.

In a recent study of the rhesus macaques, the frequency of CM CD4⁺ T cells, defined by the expression of CD28 and CD95, increased with aging (29), a finding in line with the results of our

study. The mechanism for the change in subsets of memory CD4⁺ T cells is not clear, although several possibilities are apparent. First, the increased frequency of CM CD4⁺ T cells could be a consequence of compensatory homeostatic proliferation in response to the decreased number of naïve CD4⁺ T cells. This possibility is supported by the finding that a decreased production of naïve CD4⁺ T cells occurs secondary to thymus involution in the elderly (30) and that the phenotype and function of CM CD4⁺ T cells are quite similar to those of naïve CD4⁺ T cells (12). Next, it could be secondary to a defect in the mechanism converting CM to EM CD4⁺ T cells with Ag stimulation. This latter possibility seems less likely, because at 1 wk after influenza vaccination, young and elderly subjects had a comparable level of influenza virus-specific CD4⁺ T cells producing IFN- γ and TNF- α , which were probably the combined population of pre-existing EM CD4⁺ T cells and newly generated EM CD4⁺ T cells from naïve and CM CD4⁺ T cells with the vaccination. Alternatively, the increased frequency of CM CD4⁺ T cells in the elderly could be secondary to impaired CD4⁺ T cell interactions with APCs, because a shorter T cell stimulation by APCs favors the generation of CM CD4⁺ T cells over EM CD4⁺ T cells (31). This possibility is supported by a recent study showing the decreased formation of supermolecular cluster on CD4⁺ T cells from aged mice in response to agonistic peptides presented by APCs (32), which indicates reduced T cell and APC interactions. This defect in the supermolecular cluster formation probably stems from the age-associated increase in glycoprotein expression by CD4⁺ T cells that can interrupt proper T cell and APC interactions (32). Finally, we have considered the possibility that the change in the subsets of memory CD4⁺ T cells in the elderly is secondary to alterations in the expression of IL-7 and/or its receptor, which is crucial for the survival of memory

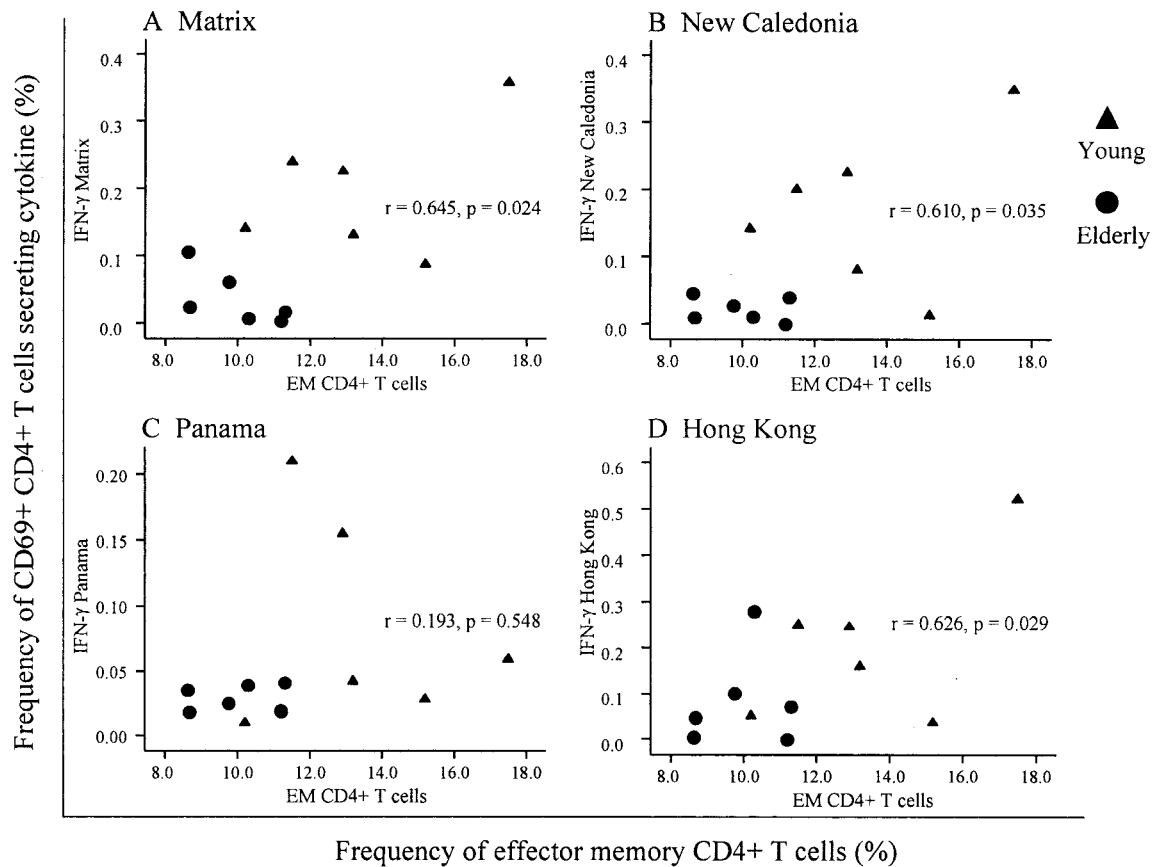


FIGURE 5. The frequency of EM (CD45RA⁻CCR7⁻) CD4⁺ T cells correlates with the frequency of influenza virus matrix protein- and influenza virus-specific CD69⁺CD4⁺ T cells secreting IFN- γ 3 mo after influenza vaccination. Peripheral blood cells from 12 subjects (six young and six elderly) who received the influenza vaccine were analyzed. Values on the x-axis are the frequency (percentage) of EM CD4⁺ T cells in peripheral blood. Values on the y-axis are the frequency (percentage) of influenza virus matrix protein- and influenza virus-specific CD69⁺CD4⁺ T cells secreting IFN- γ . The *p* values were determined using the Pearson correlation.

CD4⁺ T cells (22, 23, 33, 34). Indeed, we found lower levels of serum IL-7 in the elderly compared with the young, and a positive correlation between the level of this cytokine and the frequency of EM CD4⁺ T cells. The former finding is supported by a previous study showing decreased IL-7 expression in thymic tissues from aged mice (35). Our findings suggest that the decreased level of serum IL-7 in the elderly could be responsible for the decreased EM CD4⁺ T cells. Although the mechanism for the effect of IL-7 on T cells is not fully understood, such an effect is unlikely to be secondary to increased cell proliferation, because IL-7 does not induce the proliferation of naive and memory human CD4⁺ T cells (13, 36). Likewise, we found that IL-7 did not induce the proliferation of human CD4⁺ T cells (data not shown).

The increased frequency of CM CD4⁺ T cells in the elderly may have some benefit, because CM CD4⁺ T cells have a phenotype and function similar to those of naive CD4⁺ T cells. Thus, this subset could compensate for any possible immune deficit arising from the decreased frequency of naive CD4⁺ T cells (12). Simultaneously, CM CD4⁺ T cells can also convert to EM CD4⁺ T cells with Ag stimulation, providing the control of pathogens at the site of infection (12). However, this benefit would perhaps come at the expense of losing EM CD4⁺ T cells that are more efficient in migrating to the site of infection and producing effector cytokines compared with CM CD4⁺ T cells (12). Therefore, the altered frequency of CM and EM CD4⁺ T cells in the elderly probably contributes to the impaired CD4⁺ T cell immune responses, in particular long term EM CD4⁺ T cell responses. In our study only

peripheral blood CD4⁺ T cells were studied. Therefore, it is possible that a similar age-associated change in peripheral T cells may not occur in lymphoid tissues.

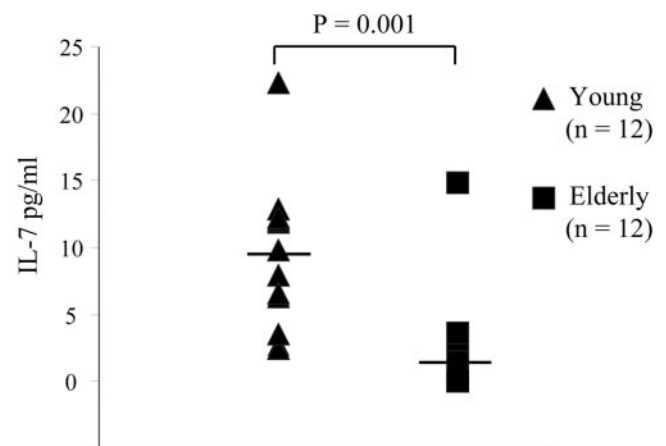


FIGURE 6. The elderly have lower levels of serum IL-7 than the young. Serum IL-7 levels were measured in 12 healthy young and 12 healthy elderly individuals using a commercially available IL-7 ELISA kit. The values on the y-axis are the concentrations (picograms per milliliter) of IL-7. The horizontal bars indicate the mean concentration. The *p* value was determined using the Mann-Whitney *U* test.

We measured CD4⁺ T cell responses specific for individual strains of influenza virus in the 2002–2003 vaccine using whole viral proteins. As far as we know, other studies have not measured CD4⁺ T cell responses to strains of influenza virus after vaccination. We detected CD4⁺ T cells specific for individual strains of influenza virus in some individuals even before influenza vaccination, indicating that the response at 1 wk was probably a composite of recently primed, as well as memory, CD4⁺ T cells. Thus, it would be somewhat difficult to reach the conclusion that the elderly have impaired naive CD4⁺ T cell responses based on our findings. We believe that such a question can be better addressed using a vaccine containing Ags to which patients have not been previously immunized. After influenza vaccination, CD4⁺ T cell responses to the strains of influenza virus and influenza virus matrix protein were variable among individuals. This indicates that the development of CD4⁺ T cell responses to individual strains of influenza virus and influenza matrix protein after vaccination is heterogeneous. However, a strong correlation was noticed between CD69⁺CD4⁺ T cells producing IFN- γ and TNF- α specific for the same strain in young and elderly subjects (data not shown), indicating that the levels of Th1-type cytokine secretion are similar for individual strains of the virus.

We observed a comparable level of humoral immune responses to the three strains of influenza virus in young and elderly individuals at 3 mo after vaccination, although a significant decline in CD4⁺ T cell responses to these strains was noticed in the elderly. Such an absence of the correlation between humoral and cellular immune responses to influenza vaccine was also reported by another group (28). These findings suggest that the declining rates of humoral and cellular immune responses may not be equal after vaccination. Studies on humoral immune responses to influenza vaccine in the elderly found conflicting results, with decreased, unchanged, and even increased responses compared with the young (37). The possible explanations for this discrepancy include differences in study subjects and in time points and methods of assessments.

We used short term stimulation assays of whole blood for the determination of influenza virus-specific T cell responses. This method appears physiologic in evaluating memory CD4⁺ T cell responses, avoiding the addition of exogenous materials and long term tissue culture (24–26). This assay has been used to evaluate HIV-, influenza virus-, CMV-, varicella zoster virus-, and EBV-specific CD4⁺ and CD8⁺ T cells (19, 24–26, 38). In these studies a difference of <0.1% in virus-specific T cells was found among patients (24–26). The mean frequency of influenza virus-specific CD69⁺CD4⁺ T cells producing IFN- γ or TNF- α (0.15%) in our study was similar to the results of other work in which the mean frequency of influenza virus-specific CD4⁺ T cells producing IFN- γ was 0.14% (19). Thus, we believe that our assays measuring activated T cells producing Th1 cytokines is a valid approach for assessment of CD4⁺ T cell immune responses after vaccination. In our study we used two different analyses, magnitude of responses (Fig. 2) and frequency of responders (Fig. 3), to evaluate influenza virus-specific CD4⁺ T cell responses using the same dataset. The results of these analyses showing the same findings provide support for the validity of our study.

The results of our study raise an important clinical question. Should the vaccination schedule and/or the dose of influenza vaccine for the elderly be altered? It is possible that a booster of influenza vaccine, which is used in the pediatric population, may increase the cellular immune responses in the elderly. Of interest, studies reported that an increased dose of influenza vaccine improved humoral immune responses to influenza virus in the elderly (39, 40). However, the impact of an increased dose and/or a

booster of influenza vaccine on the T cell immune response and the protection rate has not been evaluated.

In summary, the results of our study show that the elderly mount CD4⁺ T cell responses after vaccination comparable to those observed in younger individuals; however, they fail to maintain or expand these responses. This failure probably stems from the altered frequency of memory CD4⁺ T cell subsets, as evidenced by the increased frequency of CM CD4⁺ T cells and the decreased frequency of EM CD4⁺ T cells in the elderly. Such an alteration in the subsets of memory CD4⁺ T cells could be related to the decreased levels of serum IL-7 with aging. We believe our findings are novel because we demonstrate that alterations in serum IL-7 levels and the frequency of CM and EM CD4⁺ T cells occur with aging and that such age-associated changes potentially affect the development of long term memory CD4⁺ T cell immune responses to pathogens in vivo. These results may provide a new insight into how memory T cell immune responses change with aging.

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