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Age-Related Impaired Type 1 T Cell Responses to Influenza: Reduced Activation Ex Vivo, Decreased Expansion in CTL Culture In Vitro, and Blunted Response to Influenza Vaccination In Vivo in the Elderly

Yuping Deng,* Yu Jing,* Ann E. Campbell,† and Stefan Gravenstein*

The objective of this study was to analyze the changes in the type 1 T cell response, including the CD4+ Th1 and CD8+ T cell responses, in influenza in the elderly compared with those in young adults. PBMC activated ex vivo with influenza virus exhibited an age-related decline in type 1 T cell response, shown by the decline in the frequency of IFN-γ-secreting memory T cells specific for influenza (IFN-γ+ ISMT) using ELISPOT or intracellular cytokine staining. The reduced frequency of IFN-γ+ ISMT was accompanied by a reduced level of IFN-γ secretion per cell in elderly subjects. Tetramer staining, combined with IFN-γ ELISPOT, indicated that the decline in IFN-γ+, influenza M1-peptide-specific T cells was not due to attrition of the T cell repertoire, but, rather, to the functional loss of ISMT with age. In addition, the decline in type 1 T cell response was not due to an increase in Th2 response or defects in APCs from the elderly. The expansion of influenza-specific CD8+ T cells in CTL cultures was reduced in the elderly.

Compared with young subjects, frail elderly subjects also exhibited a blunted and somewhat delayed type 1 T cell response to influenza vaccination, which correlated positively with the reduced IgG1 subtype and the total Ab response. Taken together, these data demonstrate that there is a decline in the type 1 T cell response to influenza with age that may help explain the age-related decline in vaccine efficacy and the increases in influenza morbidity and mortality. The Journal of Immunology, 2004, 172: 3437–3446.

Influenza is a serious public health problem for people aged 50 years and older and, along with pneumonia, ranks as the fifth leading cause of death in this age group (1). In the United States alone, >10 billion dollars are spent annually on care for excess illness and hospitalization of seniors suffering from influenza, and this expense is expected to increase with the global aging of the population (2). Influenza vaccination reduces morbidity and mortality and is cost-effective for adults of all ages, but especially for elderly adults (3). For this reason, influenza vaccination is recommended for people who are >50 years of age (1). However, influenza vaccination is less effective in elderly individuals compared with their younger counterparts (4). Although the age-related decline in cell-mediated immunity (CMI) is thought to contribute to the age-related increase in influenza morbidity and mortality (5) and decline in vaccine efficacy (6), the underlying mechanism of the decline in CMI is not well understood.

A major component of CMI is type 1 T cells, which include CD8+ CTL and CD4+ Th1. Type 1 T cell responses are important in immune defenses against influenza: CTL can eliminate virus-infected cells, and the Th1 response to influenza influences B cells to secrete protective Abs. In mice, the subtypes of Abs that are most effective in neutralizing influenza viruses are IgG, particularly IgG2a, ultimately a product of a Th1 response (see Refs. 7 and 8 for review). In humans, systemic levels of IgG1 and IgG3 are important for complement fixation and Ab-dependent cellular cytotoxicity (9). IgG is the dominant Ig subclass stimulated by trivalent influenza vaccine, with IgG1 being the most prominent IgG isotype (10, 11). Although it is considered an indicator for a Th2 response in mice, IgG1 correlates with the Th1 response in humans (11–13).

An estimated 5–20% of the population is infected with influenza annually (14). Infection in elderly individuals is typically a reinfection, albeit by different, but often closely related, strains of virus, that usually stimulates a memory T cell response. Memory T cells promote an immediate and vigorous immune response specific to the challenge Ag. Unlike naive T cells, which require days to proliferate and mature into effector T cells, memory T cells exert effector function almost immediately by secreting perforin, granzymes, and cytokines such as IFN-γ and TNF-α. IFN-γ, a classical Th1 cytokine, promotes Th1 and suppresses Th2 responses. Influenza-specific memory T cells (ISMT), especially the IFN-γ+ ISMT, are vital for a rapid response to influenza reinfection (15). Virus-specific Th1 memory cells are the major source of IFN-γ, and their importance in immune response to influenza reinfections has been demonstrated in genetically altered mice (15, 16). Although aging is known to be associated with an overall increase in memory T cells and a reduction in naïve T cells, very little information is available on how aging affects the repertoire size and cellular function of ISMT.

Aging is associated with reduced influenza-specific CTL proliferation in vitro (5, 17) and reduced Ab response to influenza vaccination in vivo (17–19). The cause of these declines in CTL and Ab responses remains an open question. In the present study we seek to delineate the age-related decline in immune response to
influenza from the perspective of the frequency and the functionaltiy of ISMT. We demonstrate that there is a reduction in the frequency of IFN-γ+ ISMT in PBMC upon stimulation with influenza in the elderly. This decline is linked to the reduction in cytokine secretion, but not attrition, of ISMT with age. In addition, we show that the low frequency of IFN-γ+ ISMT is associated with a reduced growth of influenza-specific CD8+ T cells in CTL cultures. Frail elderly subjects also demonstrate a blunted type 1 T cell response to influenza vaccination that correlates directly with the Ab response, specifically the IgG1 response.

Materials and Methods

Recruitment

These studies were primarily conducted on healthy, independently living volunteers. In general, young participants were between 20 and 50 years of age, and elderly volunteers were 64 years of age or older. Subjects with egg allergy, underlying chronic diseases such as diabetes, autoimmune diseases such as systemic lupus erythematosus or rheumatoid arthritis, or congestive heart failure; those receiving ongoing treatment with immunosuppressive drugs; or pregnant women were excluded from participation. To minimize the differences in immunological background with respect to influenza, all healthy elderly subjects and their healthy young controls were vaccinated with different vaccine strains. Inoculated chicken eggs from seed viruses obtained from Caledonia/20/99 (H1N1), B/Yamanashi/166/98, and B/Victoria/2/87 were influenza A/Sydney/05/97 (H3N2), A/Panama/2007/99 (H3N2), A/New influenza and varicella zoster virus (VZV) Ag.

Influenza and varicella zoster virus (VZV) Ag

Influenza A/Sydney/05/97 (H3N2), A/Panama/2007/99 (H3N2), A/New Caledonia/20/99 (H1N1), B/Yamanashi/166/98, and B/Victoria/2/87 were generated from inoculated chicken eggs from seed viruses obtained from the Centers for Disease Control following procedures previously described (20). Brefeldin A (BFA; 5 μg/ml; Sigma-Aldrich) was added to the cells, and the incubation was continued for another 15 h. PBMC were fixed (1% paraformaldehyde; Sigma-Aldrich), permeabilized (permeabilization buffer; BD Biosciences, San Jose, CA), and stained with the following conjugated Abs: CD69-PE, CD4-allophycocyanin, CD8-PerCP, and IFN-γ-FITC (BD PharMingen, San Diego, CA). Stained PBMC were subjected to flow cytometry analysis using a dual-laser, four-color FACS Calibur (BD Biosciences, San Jose, CA). Lymphocytes (gated from scatter graph based on the relatively small size and dense population) positive for either CD4 or CD8 were designated T cells, and these T cells double positive (DP) for IFN-γ and CD69 were designated IFN-γ+ ISMT. The percentages of IFN-γ+ ISMT over total T cells were quantified and compared between young and elderly subjects. Background levels of IFN-γ+ and IFN-γ+ CD8+ T cells in PBMC by control Ags were subtracted from those of the viral Ag-activated sample, although the mock activation rarely stimulated any IFN-γ+CD8+ DP T cells that were above the detectable level (0.01% of total T cells).

Tetramer staining

PBMC (1 × 10^5 in 50 μl of complete medium) were placed in each well in a U-bottom, 96-well plate. One microliter (20 ng) of PE-conjugated M1-tetramer (HLA-A*0201 with influenza M1-peptide (GILGFVFTL) or control tetramer (HIV gag tetramer, HLA-A*0201 with HIV gag peptide SLYNTVATL; National Institute of Allergy and Infectious Diseases tetramer facility, Emory University Vaccine Center, Atlanta, GA) was added and coincubated with PBMC for 1 h at room temperature. FITC-labeled anti-CD8 Ab (Caltech, Burlingame, CA) was added, and the cells were incubated for an additional 15 min before the PBMC were washed once. The cells were immediately acquired by FACS analysis using a FACS Calibur. Results were analyzed using CellQuest software. M1-specific T cells were defined as CD8 and M1-tetramer DP cells.

ELISPOT assay

PBMC were counted using the hemocytometer and resuspended to 2 × 10^7/ml in complete medium. PBMC (150 μl, 3 × 10^7/well) were placed into each well on the top row of a 96-well plate that had been coated with anti-IFN-γ Ab (Endogen, Woburn, MA). PBMC were serially diluted in the adjacent two rows in a 1/3 titration, giving a final dilution in the third row of 1/9. There were four replicates for each dilution. After the PBMC titration, Ags were added to reach a final concentration of 10 hemagglutinin (HA) units/ml for influenza virus or a 1/8 dilution from stock for VZV Ag and VZV control Ag. Control Ags for influenza virus were either complete RPMI 1640 alone or allantoic fluid diluted in the complete RPPM 1640 to contain the same amount of egg proteins as the influenza virus Ag. PHA (2 μg/ml) stimulated PBMC were used as positive controls. For M1-peptide stimulation, 10 μg/ml M1-peptide (GILGFVFTL) was used, and the HLA-A2-restrictive HIV gag peptide (SYLNTVATL) was used as a negative control. Cells were lysed and washed away with PBS/0.1% Tween after 36 h of incubation with Ags. IFN-γ Ag captured by the plate-bound Ab was detected by biotinylated anti-IFN-γ Ab (Endogen, Woburn, MA), followed by strepavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA). Spots were developed using the blue color-forming substrate 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Kirkegaard & Perry, Gaithersburg, MD) for phosphatase. Spots were counted under a dissecting microscope.

FastImmune assay

The experimental procedures were modified from the descriptions given by Waldrop and Li (21, 22). Briefly, 1 × 10^5 PBMC were coincubated with live influenza virus (10 HA units/ml) or control Ag (either RPPM 1640 complete medium or diluted allantoic fluid) for 3 h in a U-bottom, 96-well plate. Brefeldin A (BFA; 5 μg/ml; Sigma-Aldrich) was added to the cells, and the incubation was continued for another 15 h. PBMC were fixed (1% paraformaldehyde; Sigma-Aldrich), permeabilized (permeabilization buffer; BD Biosciences, San Jose, CA), and stained with the following conjugated Abs: CD69-PE, CD4-allophycocyanin, CD8-PerCP, and IFN-γ-FITC (BD PharMingen, San Diego, CA). Stained PBMC were subjected to flow cytometry analysis using a dual-laser, four-color FACS Calibur flow cytometer, and CellQuest software (BD Biosciences, San Jose, CA). Lymphocytes (gated from scatter graph based on the relatively small size and dense population) positive for either CD4 or CD8 were designated T cells, and these T cells double positive (DP) for IFN-γ and CD69 were designated IFN-γ+ ISMT. The percentages of IFN-γ+ ISMT over total T cells were quantified and compared between young and elderly subjects. Background levels of IFN-γ+ and IFN-γ+ CD8+ T cells in PBMC by control Ags were subtracted from those of the viral Ag-activated sample, although the mock activation rarely stimulated any IFN-γ+CD8+ DP T cells that were above the detectable level (0.01% of total T cells).

In vitro generation of dendritic cells (DC)

Procedures for DC generation were similar to those described by Li et al. (22). Briefly, PBMC were adhered to tissue culture plate for 2 h. Nonadherent cells were removed, and adherent cells were cultured in the presence of M-CSF (1000 U/ml) and IL-4 (800 U/ml; BioSource, Camarillo, CA) for 7 days.

CTL culture stimulated by influenza virus

PBMC (1.5 × 10^5) in 1.5 ml of complete RPPM 1640 medium were placed in a well of a 24-well tissue culture plate. PBMC were stimulated with influenza virus A/Sydney/05/97 at a concentration of 5 HA units/ml. Cultures were incubated for 7 days, harvested, and counted. The influenza-specific CTL of these harvested cells, also called effector cells, were quantified using either FastImmune or M1-tetramer. For FastImmune, autologous PBMC infected with 10 HA units/ml of influenza virus A/Sydney (3 h) were used as APC and mixed with the effector cells at a ratio of 1:10 (APC:effector) for restimulation. APC and effector cells were coincubated for 1 h before BFA was added. Cells were incubated for another 12 h in the presence of BFA before being fixed and stained according to the FastImmune procedures described above. The percentages of CD8,
IFN-γ, and CD8 triple-positive cells were quantified. For M1-tetramer staining, effector cells were stained with M1-tetramer as described above. The percentages of M1-tetramer and CD8^+ T cells over the total lymphocytes were quantified. The total numbers of IFN-γ^+ or M1-specific CD8^+ T cells were calculated using total effector cells recovered multiplied by the percentage of the specific T cells.

Quantification of type 1 T cell response to influenza vaccination

Recruited subjects were vaccinated with 0.5 ml of the trivalent influenza vaccine (Aventis, Swiftwater, PA) during the winter season of 2001–2002. Twenty milliliters of peripheral blood was obtained before vaccination and on days 3–5, 7, 10, and 28 postvaccination for isolation of PBMC. PBMC were activated ex vivo with the combination of the three strains of virus (10 HA units/ml for each) corresponding with the vaccine strains (A/Caledonia/2099 [H1N1], A/Panama/2007/99 [H3N2], and B/Yamanashi/166/98). A stock of virus combination was prepared and frozen down in small aliquots. The same stock of virus combination was used throughout the study. Activated PBMC were stained for IFN-γ using the FastImmune procedures described above. IFN-γ and CD69 DP T cells as a percentage of the total number of CD4/8 T cells were quantified and used as the index for the type 1 T cell response. As controls, peripheral blood samples were also obtained from three unvaccinated subjects and analyzed on the same days as those from vaccinated subjects. No increase in influenza-specific type 1 T cells was observed compared with that in nonvaccinated subjects, and the SD of the ISMT detected on different days was 0.012%. Accordingly, a type 1 T cell responder was defined as having an increase in ISMT of >0.036% (3 times the SD) after vaccination.

Th1/Th2 cytokine analysis by cytometry bead array (CBA)

Supernatants from influenza virus-stimulated PBMC (same activation condition as in the FastImmune assay, except that no BFA was added) were diluted 1/1 in PBS before incubation with CBA beads coated with anti-IFN-γ, IL-2, IL-4, IL-6, IL-10, and TNF-α (human Th1/Th2 kit-2; BD Biosciences) Abs. Beads were washed and stained with PE-conjugated secondary Abs. Concentrations of each cytokine in supernatants were analyzed using CBA software (BD Biosciences).

Hemagglutination inhibition (HI) assay

The HI assay was used for determining the Ab titer specific for each of the three vaccine strains pre- and postvaccination. Five milliliters of serum was obtained immediately before vaccination and 10 and 28 days after vaccination. Serum Ab titers were analyzed by HI as previously described (23).

Human IgG subtype analysis by ELISA

Ninety-six-well plates (Immulon 1; Dynatech Laboratories, Chantilly, VA) were coated with 50 μl/well IgG subtype protein (IgG1, IgG2, and IgG3, all κ-chains; Sigma-Aldrich) as standards or with trivalent influenza vaccine 2000–2001 (A/New Caledonia/2099, H1N1, A/Panama/2007/99, H3N2, and B/Yamanashi/166/98; Aventis Pasteur, Swiftwater, PA; diluted to 1 μg of HA/ml), using coating buffer containing 0.1 M NaHCO_3, pH 8.4. After overnight incubation at 4°C, wells were washed twice with PBS with 0.05% Tween 20, pH 7.0, then blocked with 200 μl of 10% FBS/PBS in each well, incubated at 37°C for 2 h, and washed twice again. Fifty-microliter plasma samples diluted in PBS with 0.1% BSA were added to the wells and incubated for 2 h at 37°C. After four additional washes, 100 μl of biotin-conjugated anti-IgG subtype Abs (IgG1 and IgG2 (BD Biosciences; 2.5 μg/ml), IgG3 (Sigma-Aldrich; 1/4000 diluted) was added to each well and incubated for 1 h at 37°C. Plates were washed four times and 100 μl of avidin-HRP (Sigma-Aldrich; 1/4000 diluted) was added to each well and incubated at room temperature for 30 min. One hundred microliters of peroxidase substrate was added to each well after five washes. The peroxidase substrate contains 0.03% ABTS in 0.1 M citric acid plus 0.03% H_2O_2, which was added immediately before use. Color development was performed at room temperature for 45 min. Plates were read at 405 nm by a PowerWave ELISA reader (Bio-Tek Instruments, Winooski, VT).

Statistical analysis

The means of the two age groups was compared using the t test, for the sample populations that were normally distributed and with equal variances between the two groups. Normal distribution was tested using the Levene’s F test, and the modified t test was used when Levene’s F test gave a statistically significant difference between the variances of the two groups. Statistical significance was set at p < 0.05. The correlation coefficient between the two groups were analyzed using the Pearson’s correlation coefficient test.

Results

ELISPOT analysis of IFN-γ secretion ex vivo in response to influenza or VZV stimulation in young and elderly subjects

We compared IFN-γ responses to influenza virus in healthy elderly and young volunteers by quantifying the frequency of IFN-γ-secreting (IFN-γ^+ ) cells upon influenza virus stimulation using IFN-γ ELISPOT. Eighteen healthy elderly (aged 67–88 years; mean, 74 years) and six healthy young (aged 21–35 years; mean, 28 years) volunteers were studied. Elderly adults had 44% fewer IFN-γ^+ cells than young adults when PBMC were activated by influenza A/Sydney/H3N2 (p < 0.03; Fig. 1A). The IFN-γ response to different influenza subtypes (A/H1N1 or B) or strains (A/Sydney/H3N2 vs A/Panama/H3N2) was also compared in young and elderly subjects. Although the degree of activation varied (A/H3N2 > A/H1N1 > B), the age-related declines were similar (data not shown). An even greater age-related decline in the frequency of IFN-γ^+ cells was detected by IFN-γ ELISPOT when PBMC were stimulated with VZV (a 75% decline from young to elderly; p < 0.01). We also optimized the Ag presentation conditions by supplementing DC generated in vitro to the PBMC for the ELISPOT assay. The frequency of IFN-γ^+ cells increased upon addition of DC, but the age-related decline remained similar to that when no DC were added (Fig. 1B). These results demonstrate an age-related decline in IFN-γ secretion in response to influenza and VZV and show that this decline persists in the presence of DC, the professional APCs.

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** ELISPOT analysis of IFN-γ secretion in response to influenza (A) and VZV (B) stimulation ex vivo in elderly and young subjects. For the study shown in A, six healthy young (aged 21–35 years; mean, 28 years) and 18 healthy elderly (aged 67–88 years; mean, 74 years) adults were recruited. PBMC isolated from peripheral blood were stimulated by influenza A/Sydney (H3N2; 10 HA units/ml). Allantoic fluid or medium was used as a negative control, whereas PHA (2 μg/ml) was used as a positive control. For study shown in B, three healthy young (aged 22, 45, and 50 years) and three healthy elderly (aged 61, 70, and 73 years) subjects were recruited. DC were generated before the ELISPOT experiment from adherent autologous PBMC in the presence of IL-4 and GM-CSF (22). PBMC were stimulated with VZV Ag or VZV control with or without DC. DC were supplemented to PBMC in a ratio of 1:20 (DC:PBMC). Cells were cocultured with Ags for 24–36 h. IFN-γ spots were counted under a dissecting microscope. The numbers of spots were then converted to numbers per million PBMC, and the mean values from each cohort were plotted against the y-axis. The results from the PHA-stimulated PBMC were plotted against the second y-axis. Error bars are ±SE. *p < 0.03, by t test.
Flow cytometry characterization of IFN-γ-secreting cells upon influenza activation ex vivo in healthy elderly and young subjects

Although the ELISPOT assay is highly sensitive, it does not reveal the phenotype of the IFN-γ-secreting cells activated by influenza ex vivo. For this, we used the FastImmune assay, a flow cytometry-based, intracellular cytokine-staining analysis that simultaneously analyzes T cell phenotype (CD4/CD8), T cell activation marker (CD69), and cytokine (IFN-γ) secretion (21, 22). A total of five healthy young (aged 24–38 years; mean, 32 years) and 11 healthy elderly donors (aged 70–94 years; mean, 76 years) were recruited to this study. A pair of representative dot plots from the FastImmune assay of influenza-virus stimulated PBMC can be found for one young and one elderly volunteer in Fig. 2A. Fig. 2B shows the CD4+ and CD4−/8− double-negative (DN) composition among the IFN-γ and CD69 DP cells. On the average, 78 ± 13% of the IFN-γ cells were CD4+ T cells, 13 ± 4% were CD8+ T cells, and 9 ± 4% were DN cells (plausibly NK cells). No major difference was observed in CD4, CD8, and DN composition between young and elderly subjects (Fig. 2B). Flow cytometry also demonstrated that IFN-γ and CD69 DP T cells were also mostly positive for CD45R0 and CD3, the phenotype of memory T cells (Fig. 2C, a representative dot plot from the five young and 11 elderly examined in two separate experiments). Accordingly, we call these IFN-γ-cells ISMT. Elderly subjects had 48% fewer ISMT compared with young adults (p = 0.01), consistent with the result reported in Fig. 1A, and this age-related decline occurred in both CD4+ and CD8+ T cell populations (data not shown).
IFN-γ secretion level per cell from elderly subjects was also lower than that of the young subjects, as demonstrated by the 42% lower mean fluorescent intensity of IFN-γ compared with that in the young subjects (Fig. 2D; \( p = 0.04 \)). These findings indicate that the age-related decline in IFN-γ secretion upon influenza stimulation is largely accounted for by the type 1 ISMT and is characterized by a combination of fewer IFN-γ type 1 T cells and less IFN-γ secretion per cell.

**M1-specific T cells detected ex vivo by M1-tetramer and IFN-γ ELISPOT from healthy elderly and young subjects.**

To determine whether the decline in IFN-γ ISMT was due to the attrition or functional loss of ISMT with age, a cohort of healthy elderly (\( n = 20 \); aged 67–88 years; mean, 74 years) and healthy young (\( n = 9 \); aged 21–35 years; mean, 28 years) volunteers who tested HLA-A2 positive were recruited. PBMC isolated from these subjects were used to quantify the total number of M1-specific T cells by M1-tetramer and the number of M1-specific T cells capable of secreting IFN-γ by ELISPOT upon activation by the M1-peptide. The specificity of M1-tetramer staining was demonstrated by the absence of a tetramer-positive cell population in an HLA-A2-negative donor and when an HLA-A2 control tetramer (HLA-A2/HIV gag) was used for staining (Fig. 3A). Similarly, the specificity of the M1-peptide for stimulating M1-specific T cells to secrete IFN-γ was also validated (data not shown). No significant difference in the number of M1-specific CD8+ T cells was detected by the M1-tetramer comparing old and young subjects (\( p = 0.37 \)), but elderly subjects had 48% less IFN-γ+ T cells specific for M1-peptide compared with their younger counterparts (\( p = 0.013 \)), as detected by the M1-peptide-stimulated ELISPOT (Fig. 3B). Therefore, the age-related decline in type 1 T cell activation is probably due to the functional impairment of ISMT rather than to attrition of the ISMT repertoire.

**Expansions of influenza-specific CD8+ T cells in CTL cultures in elderly compared with young subjects.**

Because IFN-γ is an important cytokine promoting the growth of CTL, the age-related decline in IFN-γ secretion from ISMT may be associated with a decline in the expansion of CTL. To address this, the expansion of influenza-specific CD8+ T cells from elderly and young subjects was compared. A total of 25 healthy elderly (aged 68–87 years; mean, 77 years) and eight healthy young subjects (aged 24–37 years; mean, 32 years) were recruited for this experiment. CTL cultures of PBMC stimulated by live A/Sydney/H3N2 influenza virus were incubated for 7 days. The A/H3N2 influenza subtype was selected because it has been the predominant cause of clinical influenza for >3 decades, and it stimulates the greatest ISMT response ex vivo in both elderly and young subjects. Also, influenza A, but not influenza B, stimulated the growth of M1-specific T cells detectable by M1-tetramer (data now shown). The influenza-specific T cell frequency in PBMC was quantified immediately before and 7 days later, after CTL culture incubation, by either IFN-γ secretion or M1-tetramer staining. The M1-tetramer staining was performed on a subset of donors who tested positive for HLA-A2 (five young (aged 25–37 years; mean, 29 years) and eight elderly (aged 68–76 years; mean, 74 years)). As shown in Fig. 4A, elderly adults had fewer influenza-specific CD8+ T cells compared with younger adults, as quantified by the IFN-γ+ CD8 T cells upon reactivation of CTL culture cells. Similarly, the fold increase in M1-specific CD8+ T cells was lower in elderly compared with young subjects (Fig. 3B; \( p = 0.04 \)) despite similar frequencies of M1-specific T cells in PBMC, an observation consistent with the results reported in Fig. 3. The results demonstrate that elderly adults with a lower IFN-γ response ex vivo also have less expansion of influenza-specific CD8+ T cells in vitro, and this decreased expansion is not due to the lower precursor frequency of ISMT in PBMC.

**Ex vivo Th1 cytokines response to influenza in elderly subjects, and relationship with the Th2 response.**

We next investigated whether the age-related decline in the type 1 T cell response was due to the increase in the Th2 cytokine response. The Th2 response is of particular concern for frail elderly because it is affected by IL-6 levels, and IL-6 rises with age and disease (see Ref. 24 for review). We first recruited seven healthy young (aged 25–46 years; mean, 32 years) and 13 frail elderly (aged 81–98 years; mean age, 87 years) subjects. Using FastImmune as described in Fig. 2, a 42% decline in the frequency of IFN-γ+ ISMT was observed in the elderly compared with the young subjects (\( p < 0.03 \)). This decline in IFN-γ+ ISMT in the frail elderly was similar to that in healthy elderly subjects (Fig. 2).
To examine the possibility that the decline in IFN-γ response to influenza was due to an increase in the Th2 cytokine response, we analyzed a set of Th1 and Th2 cytokines from the supernatants of PBMC stimulated by influenza viruses from these subjects. Three Th1 (IL-2, IFN-γ, and TNF-α) and three Th2 (IL-4, IL-6, and IL-10) cytokines were quantified simultaneously by multicytokine CBA assay from supernatants harvested either 3 or 18 h after stimulation (Fig. 5). IFN-γ exhibited a significant and the largest decline at both 3 and 18 h of stimulation in the elderly frail subjects compared with the healthy young cohort (70–81% decline; \( p < 0.03 \); Fig. 5). Among the three Th2 cytokines, IL-6 was most abundantly secreted. Although the mean level of IL-6 was greater in frail elderly than in healthy young subjects, the difference did not reach statistical significance (\( p = 0.5 \)). More importantly, no negative correlation was observed between IFN-γ and IL-6 (Pearson’s \( r = 0.21 \)). IL-10 secretion was low at the beginning of activation and did not exhibit an age-related difference. At 18 h of activation, levels of IL-10 were elevated, but 63% less in elderly subjects compared with young subjects (\( p < 0.03 \)). IL-4 was close to the background level at both stimulation time points. To determine whether these results also held true in healthy elderly subjects, a similar experiment was performed in 11 healthy elderly subjects (aged 23–29 years; mean, 27 years) compared with five healthy young subjects (aged 68–80 years; mean, 74 years). The results were similar to those described in Fig. 5, with one exception; the healthy elderly had a slightly lower concentration of IL-6 compared with young subjects, but this difference in IL-6 concentration did not reach statistical significance (data not shown). These results demonstrate that the decline in type 1 T cell activation in elderly subjects is not due to the enhanced Th2 cytokine response to influenza.
we were surprised to find that the vaccine stimulated a readily detectable type 1 T cell response shown by a transient rise of IS-T1 (Fig. 6A). Among seven young and 13 elderly subjects, only one (an elderly subject) did not show any type 1 T cell response to the vaccination (see Materials and Methods for definitions of responder and nonresponder). The type 1 T cell response was detectable as early as day 5 (data not shown) and peaked on day 7 postvaccination (Fig. 6A). At the peak of the response, elderly subjects had 40.4% less IS-T1 than their younger counterparts (p < 0.03; Fig. 6B). To further compare the differences in IS-T1 response, subjects were divided into four groups: 1) nonresponders, 2) low responders with 2-3-fold rise in IS-T1 cells, 3) medium responders with 2-3-fold rise; and 4) good responders with >3-fold rise. More elderly subjects were in the low or nonresponder group, whereas fewer of them were in the good or medium responder groups compared with the young cohort (Fig. 6C). Other than the difference in the degree of IS-T1 expansion, there was a subtle difference in the kinetics of the T cell response. The T cell response in 4 of the 12 responders in the frail seniors group peaked later than 7 days postvaccination (Fig. 6D). In contrast, the T cell responses peaked on or before day 7 postvaccination for all the young subjects. These results demonstrate that frail seniors have a blunted type 1 T cell response to influenza vaccination compared with the young subjects.

The Ab response to influenza vaccination and its correlation with the type 1 T cell response were also analyzed in these two cohorts. The influenza-specific Ab response to influenza vaccination was monitored by HI assay in serum obtained before and 10 or 28 days after vaccination. The Ab response to two of three influenza virus strains was less in elderly than in young subjects, as shown by the decline in the percentage of subjects whose Ab titers either had a 4-fold rise or were at least 40 years of age (Table I). The type 1 T cell and Ab responses to vaccination were positively correlated with the frequency of IFN-γ+ T cells (see Materials and Methods for definitions of responder and nonresponder). The type 1 T cell response on day 7 and the Ab titer on day 10 (Pearson’s r = 0.82, 0.64, and 0.86, corresponding with vaccine strains of A/Panama, A/New Caledonia, and B/Victoria, respectively; Table I); this correlation was stronger for the frail elderly cohort than the healthy young cohort (Table I). Similar correlations were observed between the type 1 T cell response on day 7 and the Ab responses on day 28 postvaccination.

**FIGURE 6.** Kinetics of T cell response to influenza vaccination in frail elderly (n = 13) and healthy young (n = 7) adults, showing the reduced type 1 T cell response to influenza vaccination in the elderly. Study subjects (described in Fig. 5) were recruited and vaccinated (trivalent vaccine (2001–2002) by Aventis Pasteur, contains A/New Cledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Victoria/2/87. Twenty milliliters of peripheral blood was obtained before (day 0) and on days 3–5, 7, 10, and 28 after vaccination. As a control, three healthy donors (aged 37, 47, and 53 years) were also recruited, but were not vaccinated. PBMC were activated ex vivo, and IFN-γ-secreting cells and their phenotypes (CD69, CD4, and CD8) were analyzed by FastImmune assay similar to that described in Fig. 2. Only CD4/CD8-positive T lymphocytes were gated for analysis. A. Serial dot plots from one elderly subject who had a good type 1 T cell T cell response (100,000 events shown in each plot). The percentages of IFN-γ and CD69 DP T cells over the total T cells (CD4/CD8-positive lymphocytes) are shown in the *upper right quadrants*. B. Kinetics of type 1 T cell response to the vaccination in these two cohorts using the box graph. The *top*, *middle*, and *bottom lines* of the box correspond to the 75th, 50th, and 25th percentiles, respectively, and the whiskers extend from the 90th (upper) and 10th (bottom) percentiles. ■ Mean levels. *, p < 0.03, between elderly and young. No increase in IFN-γ+ T cells was observed in the three unvaccinated control donors over the same time course (data not shown). C. Percentages of nonresponder and low, medium, and good responders in the elderly and young cohorts. D. Peak times of the IFN-γ+ T cell response after vaccination in the two cohorts. Horizontal bars indicate the geometric means.
IMPAIRED TYPE 1 T CELL RESPONSE TO INFLUENZA IN ELDERLY

The correlation between the type 1 T cell and Ab responses was further evaluated by the response of the IgG isotype. Because influenza vaccination stimulates very little IgG4 response (25), only the IgG1, IgG2, and IgG3 responses were analyzed by ELISA. The results showed that influenza vaccination stimulated the greatest response in IgG1, whose concentrations were 10–100 times higher than those of IgG2 and IgG3 (Table II). In response to vaccination, IgG1 had the greatest increase among these three IgG isotypes. The increases in IgG1–3 were dampened in the elderly subjects compared with the young subjects, but the age-related differences did not reach statistical significance. Most importantly, the type 1 T cell response correlated positively with the IgG1 response (Pearson’s r = 0.88 and 0.74 in the elderly and young cohorts, respectively), with a less significant correlation with IgG3 in young subjects and no correlation with IgG2 in either young or elderly (Table II). These data indicate that the type 1 T cell response is associated with the IgG1 Ab response to influenza vaccination.

Discussion

Influenza can be a serious disease for elderly people whose immune function, particularly CMI, is compromised. We studied the differences in type 1 T cell responses to influenza between elderly and young adults from the perspective of the functionality of ISMT. We demonstrated an age-related decline in type 1 T cell response ex vivo to influenza in PBMC, and that this decline was not due to an attrition of ISMT, but to the loss in function of ISMT. More importantly, this age-related decline in type 1 T cell activation was associated with a reduced expansion in influenza-specific CD8\(^+\) T cells in CTL cultures in vitro. Similar to healthy elderly subjects, frail elderly also had a comparable decline in the type 1 T cell response ex vivo. In addition, the frail elderly exhibited a blunted and, in a substantial proportion, delayed type 1 T cell response to influenza vaccination in vivo, and this blunted response correlated directly with the reduced Ab response measured by both hemagglutination inhibition and IgG1 ELISA.

It is well accepted that the type 1 T cell response, particularly the CTL response, is important for combating and recovering from viral infections. Aging is known to be associated with a decline in CTL response in vitro to influenza in elderly people (5), but the mechanism of this decline is not well understood. Because an in vitro CTL response relies on the activation and proliferation of influenza-specific CD8\(^+\) memory T cells (CD8\(^+\) ISMT), which is driven by the Th1 cytokines secreted mostly from ISMT, one likely explanation for the decline in the in vitro CTL response is the age-related reduction in Th1 cytokine secretion, including IFN-\(\gamma\) secretion from CD8\(^+\) and CD4\(^+\) ISMT. Indeed, we show that aging is associated with a decline in type 1 T cell activation in response to influenza virus ex vivo in both CD4 and CD8 populations (Figs. 1 and 2), and that the decline in T cell activation is accompanied by decreased expansion in influenza-specific CD8\(^+\) T cells in CTL cultures (Fig. 4). As no age-related decline in the frequency of CTLp is observed when quantified by the M1-tetramer, we infer that it is not the number of cells that are compromised, but the functionality of the ISMT that is important (Figs. 3B and 4B). Because the majority of the IFN-\(\gamma\)-secreting cells are T cells with memory markers, the age-related decline is largely accounted for by the ISMT (Fig. 2C).

One possible explanation for our observation of the age-related decline in type 1 T cell activation is the potential dysfunction of the Ag processing and presentation by APC (mostly monocytes) in PBMC in elderly subjects. To address this, we optimized and minimized the differences in Ag processing and presentation between elderly and young subjects by supplementing autologous DC to PBMC during the IFN-\(\gamma\) ELISPOT assay of VZV-specific memory T cells. DC are the most potent APC and express high levels of costimulatory molecules as well as chemokines and cytokines that support T cell activation. Importantly, data from our group (unpublished observations) and others have shown that DCS generated in vitro using IL-4 and GM-CSF exhibit no significant age-related difference in phenotype or function (26). The results in Fig. 1B demonstrate that the age-related decline in type 1 T cell response persists in the presence of supplemented DC. Also, the age-related decline in IFN-\(\gamma\)\(^{-}\) cells in response to M1-peptide stimulation, in which the Ag processing process is bypassed, also argues against the possibility that the observed decline in the type 1 T cell response is due to the age-related dysfunction of the APC. Additional experiments rule out the possibility that the reduced T cell activation ex vivo is due to the age-related decline in B cells (data not shown). Our results favor the interpretation that there are intrinsic defects in Ag-specific, IFN-\(\gamma\)-secreting T cells. This, however, does not preclude the possibility of age-related changes in the number/function of APC in vivo.

In this study we use IFN-\(\gamma\)\(^{-}\) T cells from PBMC, briefly activated with live influenza virus, as IFN-\(\gamma\)\(^{-}\) ISMT. This ex vivo activation of PBMC is suboptimal, and therefore the frequencies of

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**Table I. Ab response to influenza vaccination and its correlation with type 1 T cell response in elderly and young subjects**

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Influenza Vaccine Strain</th>
<th>A/Panama</th>
<th>A/New Caledonia</th>
<th>B/Victoria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab geometric mean titer (pre/post)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>68.3/167.8</td>
<td>9.5/48.8</td>
<td>16.4/51.2</td>
<td></td>
</tr>
<tr>
<td>Elderly</td>
<td>14.9/70.1</td>
<td>7.3/29.0</td>
<td>17.0/66.4</td>
<td></td>
</tr>
<tr>
<td>Vaccine effective rate (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>100</td>
<td>57.1</td>
<td>71.4</td>
<td></td>
</tr>
<tr>
<td>Elderly</td>
<td>76.9</td>
<td>46.2</td>
<td>76.9</td>
<td></td>
</tr>
<tr>
<td>R (correlations between Ab and type 1 T cell response)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>0.42</td>
<td>0.68</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>Elderly</td>
<td>0.82</td>
<td>0.64</td>
<td>0.86</td>
<td></td>
</tr>
</tbody>
</table>

- \(\text{Ab response to influenza vaccination was measured by HI assay from the same elderly (}\text{n = 13}\) and young (}\text{n = 7}\) cohorts described in Fig. 6. Levels of Abs specific for these individual vaccine strains were measured from serum obtained immediately before and 10 days after vaccination. Sera were frozen down and measured simultaneously.
- \(\text{Vaccination effective rate is defined as the percentage of subjects whose influenza-specific Ab titers are } \geq 40\text{ or have a 4-fold rise after vaccination.}
- \(\text{R, Pearson’s correlation coefficient.}

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**Table II. IgG subtype responses to influenza vaccination and their correlations with type 1 T cell response in young and elderly subjects**

<table>
<thead>
<tr>
<th>Age Group</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab geometric mean (nanograms per milliliter, pre/postvaccination)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>34.1/61.8</td>
<td>3.4/4.7</td>
<td>0.4/0.5</td>
</tr>
<tr>
<td>Elderly</td>
<td>40.6/69.8</td>
<td>4.0/6.1</td>
<td>0.5/0.6</td>
</tr>
<tr>
<td>% increase (geometric mean)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>130</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Elderly</td>
<td>101</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>R (correlations with the type 1 T cell response)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>0.74</td>
<td>-0.09</td>
<td>0.63</td>
</tr>
<tr>
<td>Elderly</td>
<td>0.88</td>
<td>0.00</td>
<td>0.15</td>
</tr>
</tbody>
</table>

- \(\text{The IgG1, IgG2, and IgG3 isotype responses were analyzed by ELISA. Sera obtained immediately before and 10 days after vaccination from the 13 elderly and 7 young subjects described in Fig. 6 were analyzed. R, Pearson’s correlation coefficient.}

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IFN-γ+ ISMT are underestimated. In fact, supplementation with DC results in an increase in the detectable level of IFN-γ+, VZV-specific T cells (Fig. 1B). Furthermore, current technology is inadequate to fully quantify IFN-γ+ T cells specific for all influenza Ags without activating the bystander non-ISMT. However, the persistent decline in IFN-γ+ VZV-specific T cells with or without DC supplementation and the consistent decline in IFN-γ secretion during early and late activation (3 vs 18 h; Fig. 5) suggest that the underestimation of IFN-γ+ T cells in briefly activated PBMC does not hinder the comparison of IFN-γ+ ISMT between elderly and young subjects.

Aging, primarily due to thymic involution, is associated with an increase in the ratio of memory vs naive T cells through both an increase in the absolute numbers of memory T cells and a reduction in the output of naive T cells (see reviews in Refs. 27 and 28). Clonal expansion in both CD4 and CD8 populations further increases the absolute numbers of memory T cells with age (29). The clonal expansion is particularly prominent for CMV-specific T cells (30), although it remains unclear how aging and clonal expansion affect the repertoire size and the cellular function of memory T cells specific for other Ags. Our study does not find aging to influence the total number of M1-specific T cells, but, rather, shows a decline in the percentage that secretes IFN-γ (Fig. 3). This suggests that the observed decline in the frequency of IFN-γ+ ISMT is not due to the reduction in the repertoire size of ISMT, but, rather, reflects a qualitative change in ISMT with age.

Aging is associated with a shift from a Th1 to a Th2 response (31, 32). The IL-6 level has been reported to increase with age (24), and this increase is more prominent in elderly subjects with chronic inflammatory diseases (33). In comparing Th1 and Th2 cytokine secretion from influenza-activated PBMC, the mean level of IL-6 from frail seniors was higher than that of the healthy young and elderly controls, but it did not reach statistical significance, partially due to the large variations in the frail elderly group (Fig. 5 and data not shown). No negative correlation between IFN-γ and IL-6, the prominent Th2 cytokine in response to influenza activation, was observed, indicating that the age-related decline in IFN-γ secretion is not due to the increase in IL-6 or in the other two Th2 cytokines (IL-4 or IL-10) tested. The CBA assay also detected 70–80% less IFN-γ in the frail and healthy seniors compared with the healthy young controls. This is the reduction that our observation of a 42% decline in IFN-γ+ ISMT frequency plus a 42% decrease in IFN-γ level per cell (58 × 58% = 33.6%, or a 66.4% decline in total IFN-γ secretion) would have predicted.

To our knowledge, this is the first report of the kinetics of the type 1 T cell response to influenza vaccination in human subjects using a direct measurement of influenza-specific CD4+ Th1 and CD8+ T cells. Indirect measures of increased CTL activity to influenza after vaccination have implicated a type 1 T cell response (25). As expected, we found the split (killed) virus vaccine induces a response mostly by CD4+ Th1 T cells, with very little response by CD8+ T cells. This is because killed viruses do not replicate, and this is not optimal for Ag processing and presentation of CD8+ T cell-restrictive Ags (>90% of the ISMT detected at the peak of the response to influenza vaccination are CD4+ T cells; data not shown). In naturally infected individuals, a strong CD8+ T cell expansion was detected using the same methods for detecting the type 1 T cell response to influenza vaccination (our unpublished observation), suggesting that the low CD8+ T cell response to influenza vaccination as well as the relatively low percentage of CD8+ T cells among all IFN-γ+ T cells in influenza-activated PBMC are not due to the limitations of the assay per se.

Aging is associated with a general decline in Ab response, and this decline can be largely attributed to the senescence of Th cell function (34). In this study we observe a positive correlation between the IFN-γ+ T cells and the Ab response, and this correlation is demonstrated in both healthy young subjects and frail elderly subjects (Tables I and II). A similar positive correlation between the IFN-γ secretion and the Ab response is reported by Murasko and colleagues in a study that involved a large cohort of healthy elderly (n = 233) (35). In that study, the level of IFN-γ-secretion from PBMC obtained after vaccination in response to influenza vaccine correlates positively with the Ab response measured by HI assay (35). Our finding of the positive correlation between the type 1 T cell and IgG1 responses in our study is intriguing. IgG1 is used as an indicator for a Th1 response in mice. However, IgG1 have been shown to be associated with a Th1 response in many independent studies in humans (11–13). Our results further support the idea that IgG1 is an indicator for a Th1, rather than a Th2, response in humans.

It remains to be examined whether the age-related decline in the type 1 T cell response as well as the positive correlation between the type 1 T cell and Ab responses also hold true in healthy elderly subjects. The status of the immune system in some frail elderly could be sufficiently compromised so that their type 1 T cell and Ab responses are different from those of healthy elderly subjects. However, because disease prevalence increases with age, healthy elderly represent <15% of the general older adult population (36). Restricting the study subjects to only healthy individuals simplifies the data interpretation, but limits its application to the larger population of interest. Frail older subjects are also at greater risk from influenza and influenza-related complications, increasing their need for vaccine-derived protection and potential benefit. Data regarding vaccine immunogenicity in this population to inform us about how to improve vaccines remain limited, but are therefore very relevant.

An age-related decline in the type 1 T cell response to respiratory syncytial virus was reported recently, measured as respiratory syncytial virus-activated PBMC using IFN-γ ELISPOT (37). This is consistent with our observation of the age-related decline in the type 1 T cell responses to influenza and VZV. However, no age-related difference in the type 1 T cell response to human CMV was observed (our unpublished observation). In addition, PHA stimulation did not show an age-related difference in IFN-γ secretion from T cells (Fig. 1A). Therefore, the age-related decline in type 1 T cell response to influenza Ag cannot be generalized to all other viral Ags, nor is the decline indicative of the overall status of the CMI. This underscores the importance of focusing a particular population of Ag-specific T cells, instead of a mixture of them by using nonspecific stimuli such as anti-CD3 Ab or PHA, when studying the age-related changes in the immune system.

There are many possibilities leading to the age-related decline in the type 1 T cell response to influenza. One obvious possibility is the different immunological backgrounds, such as the history of acute influenza infection or vaccination. Elderly adults will have an exposure history with a variety of related influenza viruses over many decades of influenza seasons, whereas younger adults will have a limited exposure history. As such, the ISMT pool may differently represent this history and influence the response to future challenge, such as by vaccination. In addition to the immunologic background, the microenvironment (such as cytokines, level of oxidative stress) can also contribute to functional changes in ISMT. Re-exposure to Ag due to reactivation of pathogens (VZV) or reinflection (such as influenza) can further affect the quantity and quality of Ag-specific memory T cells (38). Because aging is also known to be associated with a decline in the ability to generate effector T cells (39), it is likely that an infection in the elderly may produce fewer and inferior memory T cells compared...
with that in young adults. The underlying mechanism of this age-related decline in the functionality of type 1 memory T cells is complex and requires further study. Understanding the mechanisms of the age-related decline in CMI will offer insights for new vaccine designs for elderly populations.

In summary, our work demonstrates an age-related decline in type 1 T cell activation in response to both replicating and non-replicating influenza stimulation. In the populations we studied, the decline in type 1 T cell response is due to the functional decline in ISMRT rather than to attrition of the T cell repertoire, defects in Ag processing and presentation, or the suppressive effect of an age-related increase in Th2 cytokines. Blunted and some delayed-type 1 T cell responses to influenza vaccination are also observed in frail elderly compared with young subjects. We believe that it is this decline with age that hampers the CTL response to acute influenza infection and also contributes to the reduced Ab response, at least the Th1 cytokine-driven component of the Ab response, to influenza vaccination. These observations help explain the age-related increase in influenza morbidity and mortality as well as the age-related decline in vaccine efficacy.

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