Published in final edited form as:
*Vaccine*. 2011 April 5; 29(16): 2865–2873. doi:10.1016/j.vaccine.2011.02.017.

# Antibody and Th1-type cell-mediated immune responses in elderly and young adults immunized with the standard or a high dose influenza vaccine

**Wilbur H. Chen**[1,2,*], **Alan S. Cross**[1,2], **Robert Edelman**[1,2], **Marcelo B. Sztein**[1,3], **William C. Blackwelder**[1,2], and **Marcela F. Pasetti**[1,3,*]

[1] Center for Vaccine Development, University of Maryland School of Medicine, Baltimore MD 21201

[2] Division of Geographic Medicine, Department of Medicine, University of Maryland School of Medicine, Baltimore MD 21201

[3] Division of Infectious Diseases and Tropical Pediatrics, Department of Pediatrics, University of Maryland School of Medicine, Baltimore MD 21201

## Abstract

A comparative analysis of antibody and cell-mediated immune responses was performed in ambulatory medically stable elderly and young adults who received the standard-dose of trivalent inactivated influenza vaccine, containing 15 μg of hemagglutinin (HA) per virus strain, or a high-dose vaccine containing 60 μg HA per virus strain. Among the elderly, the high dose vaccine induced greater HAI (hemagglutination inhibition) and virus neutralization antibody titers than the standard dose vaccine. These responses, however, did not achieve the magnitude of those induced by the standard dose vaccine in young adults. Vaccine-specific circulating T cells producing IFN-γ were detected in the elderly and young adults following immunization. However, there were no significant differences in the IFN-γ responses among groups. On the other hand, the standard dose vaccine in the elderly resulted in the highest proportion of complete non-responders who failed to elicit either an HAI or an IFN-γ response. This study provides further evidence that a higher dose vaccine for the elderly may result in enhanced immune responses which are predicted to improve protection although still of lower magnitude than those induced in younger healthier individuals.

### Keywords

Influenza; vaccines; elderly; immune responses

[*]Corresponding Authors: Wilbur H. Chen, M.D., M.S.; Marcela F. Pasetti, Ph.D., Address: 685 W. Baltimore Street, Suite 480, Baltimore, MD 21201, Tel: 410-706-5328, Fax: 410-706-6205, wchen@medicine.umaryland.edu, mpasetti@medicine.umaryland.edu.

**Potential conflicts of interests:** W.H.C. has been a consultant for AlphaVax, LigoCyte Pharmaceuticals, and Toyama Chemical Co. R.E. is a consultant for Sanofi Pasteur.

## 1. INTRODUCTION

Influenza virus infections represent one of the leading causes of morbidity and mortality in the elderly; >90% of annual influenza-associated mortality occurs among those 65 years of age and older [1]. Although annual vaccination is the primary method of preventing influenza infection, the effectiveness of the currently approved influenza vaccines in the elderly is lower than in healthy young adults [2–6]. One explanation for the poor responses to the vaccine among the elderly is a phenomenon termed immunesenescence, which is described as a progressive, slow and steady decline in the function of the immune system during aging [7].

Virus-specific antibodies measured by hemagglutination inhibition (HAI) have been traditionally associated with protective immunity against influenza [8]. Although assessments of antibody responses to influenza vaccines in the elderly has yielded conflicting results [9], aging has been associated with reduced hemagglutinin (HA) antibody [10] as well as reduced cell-mediated immune (CMI) responses to influenza vaccines [11].

One promising approach to improve the protection afforded by influenza vaccines in older adults is to increase the amount of antigen contained in each vaccine dose. Until recently, influenza vaccines approved for use in the elderly contained 15 μg of HA from each of three annually selected virus strains. However, a high dose influenza vaccine (60 μg of HA/virus strain) was approved for use in adults age ≥ 65 years on December 23, 2009. Several studies have shown that vaccine formulations containing higher dosage levels of HA can be administered safely and induced greater HAI antibody responses in the elderly [12–19].

A recent multi-site influenza Phase 2 clinical trial in elderly adults, in which our group participated, showed significantly higher HAI and virus neutralization (VN) antibodies for all three vaccine virus strains among recipients of the high dose influenza vaccine in comparison to those that received the standard dose [20]. An important aspect that was not addressed in that study, however, was the how the responses induced by the influenza vaccine in the elderly compare in both quality and magnitude with those of young healthy adults, particularly whether the high dose vaccine is able to achieve the levels of responses elicited by young adults.

We report here the immunogenicity of a trivalent, inactivated influenza vaccine administered to healthy ambulatory elderly adults using the standard dose (15 μg HA of each virus strain) or a high-dose (60 μg HA per virus strain) formulation, in comparison to young adults who received the standard-dose vaccine. The immunological outcome measures were HAI, VN, and HA-specific serum IgG and IgA levels. We also investigated the induction of CMI by measuring the frequency of IFN-γ-secreting T cells in peripheral blood.

## 2. MATERIALS AND METHODS

### 2.1. Participants and study design

Forty-nine healthy, independently living elderly (≥65 years) volunteers participating in a multi-center influenza vaccine trial [21] during April 11–22, 2005 agreed to participate in a substudy (described herein) to further characterize the immune responses induced by the trivalent inactivated split-virus influenza vaccine (TIV) administered in the standard or an experimental high-dose formulation. For comparison, 15 young adults (18–40 years) were enrolled and vaccinated, under an independent complimentary protocol, with the standard-dose TIV during June 22–29, 2005. Prior to vaccination, each subject was screened for medical stability and excluded if the following applied: presence or history of malignancy, immunosuppression or use of a potentially immunosuppressive medication, receipt of blood

product within the past 3 months, autoimmunity, acute or chronic medical condition that might adversely affect immune responses (such as chronic liver disease, significant renal disease or heart failure, diabetes mellitus, organ transplant recipients), or pregnancy. The day of vaccination, vital signs were recorded and a negative pregnancy urine test was requested from females of child-bearing potential.

### 2.2. Vaccines

The vaccines administered were 1) the licensed Sanofi Pasteur (Fluzone®) 2004–2005 TIV containing 15 μg of HA of A/New Caledonia/20/99 (H1N1), A/Wyoming/03/2003 (H3N2), and B/Jiangsu/10/2003 and 2) an experimental high-dose vaccine prepared in a similar manner but containing 60 μg of HA for each of the same viral strains; the dose volume for both vaccines was 0.5 ml [22]. The elderly cohort was randomly allocated to receive either the standard or the experimental high-dose vaccine in a double-blinded fashion. The young adult cohort received the standard-dose vaccine (Fluzone®). All subjects provided their informed consent to participate in the study. The protocol was approved by the Institutional Review Board of the University of Maryland, Baltimore and registered in clinicaltrials.gov as NCT00115531 and NCT00170508.

### 2.3. Clinical Samples

Blood was obtained prior to vaccination (Day 0) and 4 weeks later (Day 28). Cell blood counts with differentials were performed the day of vaccination. Serum and peripheral blood mononuclear cells (PBMC) were obtained at both time points for serological and CMI assays, as previously described [23].

### 2.4. Antibodies

Seed viruses matching the vaccine strains were kindly provided by Dr. Jacqueline Katz, CDC Influenza Branch, and virus stocks were produced in embryonated chicken eggs, as previously described [24]. HAI antibody levels to the 3 vaccine components were measured by incubating serially diluted serum samples (starting at 1:4) with 4 HA Units of each antigen and chicken erythrocytes, following standard techniques [25]. Sera were pre-treated with receptor destroying enzyme (Denka Seiken Co. Tokyo, Japan) to inactivate nonspecific inhibitors of viral hemagglutination [26]. HAI titers were calculated as the inverse of the highest dilution that inhibited hemagglutination. Viral neutralization titers were measured as previously described [24]. Briefly, heat-inactivated 2-fold diluted serum samples were mixed with each virus (100 $TCID_{50}$) and added to MDCK cell monolayers in 48-well flat bottom plates (Nunclon, Rochester, NY). The cytopathogenic effect (CPE) was recorded over the next three days and the end point titer for each sample was calculated as the inverse of the highest dilution that completely abrogated virus CPE. Virus-specific IgA, IgG and IgG subclasses were measured by ELISA. Briefly, 96-well microtiter plates were coated with each influenza antigen (prepared in embryonated eggs) for 3 h at 37°C and blocked overnight with 10% non-fat dry milk (Carnation) in PBS. Serially diluted serum samples (starting at 1:50) were added and incubated for 1 h at 37°C. Plates were then washed with PBS-Tween 0.05% and incubated with HRP-labeled goat anti-human IgG (Jackson ImmunoResearch Labs, West Grove, PA); anti-IgA (MP Biomedicals, Solon, OH); or sheep anti-human IgG1, IgG2, IgG3, or IgG4 (The Binding Site, San Diego, CA). Incubation with the substrate 3, 3′, 5, 5′ tetramethyl benzidine (TMB; KPL labs, Gaithersburg, MD) was 15 min before stopping the reaction with the addition of 100 μl of $H_3PO_4$. End point titers were calculated from linear regression curves as the inverse of the dilution that produces an $Absorbance_{450}$ of 0.2 above the blank. Samples were tested in duplicate in conjunction with calibrated controls. IgG avidity was measured by ELISA, as described above, with an additional chaotrope elution step [27]. After incubation with the samples, plates were washed once with PBS-Tween 0.05% and incubated with 200 μl of 6M urea for 10 min,

followed by four additional washes. The strength of antibody binding was reported as Avidity Index (AI) and calculated as the ratio between IgG titers in the presence and absence of Urea × 100.

### 2.5. IFN-γ ELISpot

The frequency of IFN-γ-secreting cells was measured by ELISpot, as previously described [28]. Briefly, cells thawed and rested overnight in complete medium (RPMI 1640 supplemented with 100 μg/ml penicillin and streptomycin, 2 mM L-glutamine, 2.5 mM sodium pyruvate, 10 mM HEPES buffer, and 10% heat-inactivated FCS) were added to nitrocellulose plates previously coated with anti-human IFN-γ mAb (5 μg/ml, clone 2G1; Endogen, Woburn, MA) and incubated with the dialyzed influenza vaccine at 0.5 μg/ml, 1 μg/ml, and 2 μg/ml for 16 h at 37°C, 5% $CO_2$. Samples were tested in triplicate in 2-fold dilutions (100,000–12,500 cells/well). Negative control wells contained cells alone or keyhole limpet hemocyanin (KLH 1 μg/ml, Pierce). Positive control wells contained CD3/CD28 beads (0.6 μl/ml, Dynal). Plates were then washed and incubated with biotin-labeled anti-human IFN-γ mAb (2 μg/ml, clone B133.5, Endogen) followed by avidin-peroxidase (Kirkegaard & Perry Labs). TrueBlue was used as the detection substrate (Kirkegaard & Perry Labs). Spots were counted in an automated ELISpot reader (Bioreader 3000 PRO, Bio-Sys, Karben, Germany). Net frequencies of IFN-γ spot forming cells (SFC) were calculated as SFC in experimental wells –SFC in negative control wells. The cut-off for a positive response was 53 SFC per $10^6$ PBMC, and it was established as the mean frequency of IFN-γ SFC from all patients and time points cultured with KLH + 3 S.E. [29]

### 2.6. Statistical Analysis

Antibody geometric mean titers (GMT) were calculated using log-transformed data. Seroconversion was defined as a 4-fold increase in antibody titer at day 28 over the pre-vaccination value. For IFN-γ SFC, a response was defined as a 2-fold increase at day 28 over the baseline, with a pre-vaccination IFN-γ SFC value of 0 set to 1. Proportions of IFN-γ responders were compared by chi-square test. The absolute increase in IFN-γ SFC was considered to be 0 if the post-vaccination value was less than the pre-vaccination value. The logarithm of fold-increase in antibody, absolute increase in IFN-γ SFC response after vaccination, and absolute increase in IgG subclass response were analyzed by two-sided Wilcoxon signed-rank test (exact test or, in case of ties, normal approximation test with continuity adjustment). For post-vaccination antibody responses, logarithms of titers in different vaccine groups were compared overall by one-way analysis of variance (ANOVA) and pairwise using the Tukey-Kramer multiple comparisons procedure. For fold increase, since residuals often were not fit well by a normal distribution, data were compared using Kruskal-Wallis ANOVA with adjustment for ties (overall) and the Dunn multiple comparisons procedure for ranks. A p-value $< 0.05$ (two-sided, when comparing two groups) was considered statistically significant. In order to maintain the overall significance level at 0.05 in the Dunn procedure, each of the three individual comparisons was required to have $p < 1 - 0.95^{1/3} = 0.01695$ to be considered significant.

Differences in HAI and VN antibody titers between groups were also assessed separately using linear regression models for log-transformed titers that included variables for prior vaccination and interactions between vaccine groups and vaccination during the previous season. Analyses were performed using NCSS 2007 (Kaysville, UT) and GraphPad Prism 4.0 (La Jolla, CA).

# 3. RESULTS

## 3.1. Demographics

The demographic characteristics of the vaccine recipients are summarized in Table 1. Forty-nine medically stable elderly volunteers (65–85 years, mean age 74 years) were enrolled in the study (no subjects were terminated early); 25 were male and all elderly subjects were white, non-Hispanic. Twenty-six elderly subjects received the standard-dose vaccine (ES cohort) and 23 received the high-dose vaccine (EH cohort) (Figure 1). Fifteen medically stable young adult volunteers (20–40 years, mean age 24 years) were enrolled; 9 were male, 10 White, 1 Black, 4 Asian, and none were Hispanic. However, one young adult participant was excluded from the study based on a low total lymphocyte and high eosinophil count on the pre-vaccination blood draw. It was subsequently discovered that this person had a recent history of systemic steroids treatment for an asthmatic flare (an exclusion criterion denied by the volunteer during medical history screening). All young adults received the standard-dose vaccine (YS cohort) (Figure 1). The majority of elderly (21 in the ES and 18 in the EH cohort) and a minority of young participants (4 in the YS cohort) received influenza vaccination the previous influenza season; nearly all these vaccines were administered October–November of 2004 and none were received after December 2004.

## 3.2. Antibody responses

Serum HAI titers are traditionally used as a surrogate measurement of influenza vaccine efficacy. An HAI titer of 1:32 is commonly accepted as the threshold of protection, termed “seroprotection,” and higher titers correlate with reduced likelihood of infection. The HAI antibody responses against the 3 virus strains contained in the vaccines were measured in all participants before and after vaccination. The YS group demonstrated the highest proportion of subjects that achieved seroprotection (titer ≥1:32) or seroconversion (≥ 4-fold increase in titer over baseline) to each of the three strains (Table 2). The mean fold-increase in antibody titers after vaccination as well as the p-values comparing pre- and post-vaccine responses in the elderly vs. young adults are shown in Figure 2. The YS group consistently produced the most robust HAI responses against each of the three viruses, by both fold-increase in titers and GMT. In contrast, the ES group had the lowest HAI responses of all groups for all three viruses, failing to show more than a 2-fold rise in post-vaccination GMT. Even though the HAI titers in the EH cohort were higher than those of the ES group, they did not reach levels achieved by the YS cohort. While the median fold-increases in HAI titers were not statistically significantly different between the EH and YS groups, the post-vaccination GMT for H1N1 and H3N2 were significantly higher in the YS cohort compared to either of the other groups. There was no significant difference in the HAI responses to the B virus between EH and YS groups, either by fold increase or by GMT. Linear regression analyses of log-transformed HAI post-vaccination antibody titers to H1N1, H3N2, and B virus showed that when there was no prior year vaccination, titers were on the average higher in the YS group compared with the ES ($p<0.0001$, $p=0.003$, and $p=0.018$, respectively), and in the EH group compared with ES ($p=0.008$, 0.041, and 0.020, respectively). Titers were significantly higher in the YS group than in EH for H1N1 ($p=0.046$) but not for H3N2 ($p=0.51$) or B ($p=0.75$). In subjects who received influenza vaccine the previous season, titers in the YS group were significantly higher than in ES for H1N1 ($p=0.025$) but not for H3N2 ($p=0.53$) or B ($p=0.47$). There was a trend toward higher titers in the YS group than in EH for H1N1 ($p=0.058$) but not for H3N2 ($p=0.34$) or B (0.82); there were no significant differences between titers in the EH and ES groups ($p\geq0.10$ for all antigens).

To further assess the effect of age and vaccine dose on the functional capacity of the antibodies produced, we measured influenza VN titers in all participants before and after vaccination. Figure 3 shows the VN antibody responses against the 3 viral strains as well as

the mean fold-increases in antibody titers and p values comparing pre- and post-vaccine responses. As observed for the HAI antibodies, the YS group exhibited the highest VN titers for each of the three influenza strains. The ES group had the lowest responses of all three groups, without achieving a greater than 2-fold rise above baseline for any of the strains. Responses in the EH group were slightly above those of the ES cohort but did not exceed those of the YS cohort. The VN titers for the H1N1 and B viruses were not significantly different between YS and EH by either fold-increase or post-vaccination GMT. Interestingly, for the B virus, higher pre-vaccination GMT were seen in both elderly groups compared with the YS group, probably due to exposure to natural infection or previous vaccinations. Results from regression analyses of log-transformed post-vaccination VN titers for subjects with no influenza vaccination the previous season showed significantly higher titers to H1N1 and H3N2 in the YS group compared with the ES ($p = 0.0007$ and 0.0001, respectively) but not to B ($p=0.29$). Titers against H1N1 and H3N2 were also higher in the YS group compared to the EH, but not significantly ($p=0.11$ and $p=0.13$, respectively). In contrast, the EH group had higher responses to B compared to the YS ($p=0.065$). The EH group had higher average titers than the ES for all three strains (H1N1, H3N2, and B, $p=0.10$, 0.028, 0.013, respectively). There were no significant differences between groups for any antigen in subjects who received influenza vaccine the previous season, ($p \geq 0.16$ for all comparisons).

To further examine how age and vaccine dosage might influence the antibody response, the strain-specific serum IgG responses were measured by ELISA in all vaccinated subjects (Figure 4). As with the HAI and VN antibody responses, the YS group demonstrated the highest responses in strain-specific IgG, whereas the ES group had the lowest. None of the subjects in the ES group responded by a four-fold rise in ELISA IgG to any of the three virus strains. The IgG responses in the EH group slightly surpassed those of the ES cohort, but remained lower than those of the YS group.

We hypothesized that analysis of IgG subclass distribution and avidity might provide insights into the quality of the antibody responses to influenza vaccination in the elderly vs. young adults. Hence, we next examined the IgG subclasses and IgG avidity in subjects that showed a positive response to the vaccine (i.e. had a 4-fold increase in serum IgG over baseline). Among the YS and EH IgG responders, the IgG1 subclass was the most prevalent, with elevated titers for all 3 virus strains (Figure 5). Only the YS group demonstrated IgG1 subclass responses with statistically significant absolute increases due to vaccination; there were too few responders in the EH group to show significant IgG1 subclass increases. IgG2 was also detected in the YS and EH cohorts, albeit at much lower levels; no significant increases were seen after vaccination. The levels of IgG3 and IgG4 were almost negligible.

We did not observe an increase in IgG avidity following vaccination, most likely due to the limited timing of sample collection (1 month after vaccination). Overall, the mean avidity index was lower for the YS group compared with the EH group for the two strains (H3N2 and B) with available data (data not shown).

To examine whether the differences in antibody responses also extended to other immunoglobulins, we measured influenza-specific IgA titers following vaccination. The highest responses (by GMT and fold-increase) were detected in the YS group, whereas modest responses were seen in the ES and EH cohorts (less than a 2-fold increase in titers). Among the YS subjects, 50% (7 of 14) demonstrated seroconversion (four-fold increase) to H1N1, and 14% (2 of 14) seroconverted for the H3N2 and B strains. None of the ES subjects demonstrated seroconversion to any of the 3 strains. There were, however, a few individuals among the young and one EH subject (to the B strain) who exhibited extremely high virus-specific IgA responses (data not shown).

### 3.3. Th1-type cell-mediated immunity

Given the likely contribution of CMI to protection from influenza, we also examined the effect of aging and vaccine dose on influenza-specific T cell responses. To this end, we measured the frequency of IFN-γ secreting cells by ELISpot in peripheral blood following ex vivo antigen stimulation among 15 randomly selected ES subjects, 15 EH subjects and 14 YS. Figure 6 shows the IFN-γ SFC responses before and after vaccination; data represent peak responses from 3 different antigen concentrations tested. An average 2-fold increase in the frequency of IFN-γ SFC was seen in all 3 groups following vaccination. We also observed statistically significant absolute increases in IFN-γ production elicited by immunization in all three cohorts. When defining IFN-γ responders as those individuals who had at least a two-fold increase in SFC above the threshold, we observed 57% responders in YS (8 of 14), 47% EH responders (7 of 15), and 47% ES responders (7 of 15). These response rates were not significantly different ($p = 0.81$).

### 3.4. Analysis of combined antibody and CMI responses

The ability to elicit both a humoral and CMI response following vaccination is likely critical for optimal protection against infection, We defined an antibody response as a ≥ 4-fold rise plus a titer ≥ 1:32 post-vaccination for at least one virus strain by HAI. A positive CMI response was defined as a positive IFN-γ SFC response (> cut off) and ≥ 2-fold increase post-vaccination. The number of subjects with “any response” (either antibody or CMI) was 14 in the YS cohort, 8 in the ES cohort, and 12 in the EH cohort. When examining the proportion of subjects with a “combined response” (both antibody and CMI), there were 7 in the YS cohort, 1 in the ES, and 4 in the EH cohort; only a significant difference between the YS and ES groups ($p=0.014$ by two-tailed Fisher’s Exact Test). In pairwise comparisons by two-sided Fisher exact test, none of the differences in proportion of combined responders was significant.

We defined “complete non-responders” as those who failed to elicit either an antibody or CMI response. There were no complete non-responders in the YS cohort, 7 in the ES cohort, 3 in the EH cohort; the difference in proportions of complete non-responders was significant between the YS and ES groups ($p=0.006$) but not between the YS and EH or ES and EH groups, by two-sided Fisher exact test.

## 4. DISCUSSION

The public health importance of the effects of aging on influenza vaccine responses has become increasingly more evident as the global elderly population continues to dramatically rise. Development of effective influenza vaccines targeted specifically to this high-risk group remains a high priority. The protection elicited by a successful influenza vaccine for the elderly will likely need both humoral and cellular-mediated immunity, requiring full engagement of the host immune system. However, during aging the coordination and regulation of immunological functions are impaired as a result of immunesenescence [30;31].

In this study, we identified significant differences in the immune response to an inactivated subunit influenza vaccine between seniors and young adults. We also determined the extent to which a four-fold higher dosage vaccine might enhance the immune response despite these age-related immunological defects. Our results showed that there was a clear age-related impairment in the HAI and VN antibody responses to the standard-dose influenza vaccine for each of the three vaccine virus strains. Although the high-dosage vaccine improved antibody responses measured by GMT, fold-increase in titers over baseline, and seroconversion and seroprotection rates in elderly subjects, this group was unable to attain

the magnitude of responses elicited by the young adults who received the standard-dose vaccine. The relatively small sample sizes of the groups, might have underpowered the study, limiting our capacity to detect statistically significant differences for some of the immunological outcomes. Because elderly patients are usually under medication or have underlying health conditions, recruitment of participants that met the study requirements was difficult. Nonetheless, the HAI results from the parent Phase 2 trial [32], which was a multi-site study that included a much larger number of elderly subjects, were similar to those of the elderly group reported here, and yet still failed to exceed the HAI responses of the YS, by both GMT and fold-rise in serum levels. Because we studied a healthy and active elderly population as opposed to senior community subjects in the parent study, our data support the notion of a general decline in the immune capacity of medically stable, active elderly people and challenges the concept that the poor immune responses of the elderly are due to reduced health status [33]. In previous studies of multivalent higher dosage influenza vaccines, increases in antibody levels were not observed for all the components [34–36].

A limitation when interpreting our data is the vaccination history of the subjects enrolled, since prior exposure to influenza vaccine can influence responses to a subsequent immunization [37–39]. It is also possible that some of the discrepancy between our data and the immune responses reported previously by others, result from differences in priming background. The enrollment criteria of the parent study that recruited the elderly subjects did not exclude participants based on prior vaccination history; in fact it sought a broader inclusion to obtain data representative of the general population. In that study, the responses of elderly subjects with a recent prior influenza vaccination were found to be lower than those of elderly without recent vaccination [40]. The majority of the elderly subjects we examined received influenza vaccination 5–6 months prior to our immunization, during which the same 3 virus strains were administered (Table 2B). Conversely, a minority of the young individuals received influenza vaccination during the same time period. When we examined our data adjusting for prior immunization through multiple linear regression analysis we found superior antibody responses in the YS group compared with the elderly for most of the virus strains. However, due to the low numbers of subjects in some of the groups, we failed to demonstrate statistiscally significant differences in all comparisons. The small number of subjects also precluded us from further dissecting the influence of priming history on the immune responses in relation to age.

Several studies have examined the effect of higher dosage levels of influenza vaccine on the elderly [16;41–44]. Only one of these studies attempted to compare these responses to those of healthy young adults [16], whereas none of them examined CMI responses.

Because a previous report had suggested that ELISA might be superior to HAI in distinguishing differences to influenza vaccination [45], we further explored the humoral immune responses by examining influenza-specific IgG, IgA, IgG subclasses and avidity by ELISA. The serum IgG and IgA antibody responses of the elderly were lower than those of the young; the high-dose vaccine resulted in some improvement of those immune responses but still fell short of those induced by the standard dose in young adults. In contrast, none of the elderly subjects that received the standard-dose vaccine achieved a four-fold rise in IgG antibodies, stressing again the need for a higher dose of antigen to trigger a stronger response during immunesenesence.

Aging has been shown to be associated with a significant impairment of IgG1, but not of IgG3 [46]. We did not find significant differences in the IgG subclasses between the young and elderly subjects in response to influenza vaccination. IgG1 was the main subclass produced, followed by IgG2, in both groups and for all strains. This increase in IgG1 levels following immunization, particularly in the EH group, may reflect a predominant Th2-type

response. A major limitation of our study was the low number of subjects that could be included in this analysis. Nevertheless, the trends were consistent and clear. In addition, we were unable to provide evidence for or against a defect in avidity maturation as another feature of age-related effect on vaccination [47]. It is possible that IgG subclass distribution and avidity are more dependent on priming and boosting by natural infection or vaccination rather than on age [48;49].

Interferon-γ production and other CMI responses may represent complementary and relevant effector mechanisms involved in protection against influenza, especially in the elderly. Although we were unable to demonstrate significant differences in IFN-γ production among the age and dose groups, there was a trend for the young to have higher post-vaccination IFN-γ peak SFC. We did not observe improved SFC in the higher dose vaccine group among the elderly. These findings seem to support the view that there are significant age-related defects in lymphocyte function and/or Th2-biased responses [50–52]. The absence of an IFN-γ response may reflect a Th2-type response to this vaccine, although an association was not examined. Notably, we observed significant or almost significant increases in vaccine-induced IFN-γ production in all groups of volunteers following immunization, suggesting that the elderly still maintain the ability to respond to TIV vaccination with CMI responses of moderate magnitude. The IFN-γ production described in our study most likely results from vaccine-primed $CD4^+$ Th1 cells, with limited contribution of $CD8^+$ T cells. We are further exploring the potential for T cell responses in these subjects, including the involvement of specific $CD4^+$ and $CD8^+$ subsets and the activation of vaccine-specific effector and memory T cells. The highest risk of influenza disease was previously demonstrated to be among individuals that had neither a humoral (HAI) or CMI response to vaccination [53]. We showed that immunization of the elderly with the standard-dose vaccine was associated with high numbers of complete non-responders (i.e., individuals lacking both antibodies and CMI) compared with immunization with the high-dose vaccine. In contrast, none of the young adults receiving the standard-dose were complete non-responders.

In conclusion, there is an urgent need to develop newer generation influenza vaccines to specifically address the limitations of the current vaccines in protecting elderly people. Our study shows a clear age-associated impairment in host immunity against influenza vaccination and an improvement of humoral responses with the use of a higher dose vaccine. The superior antibody levels induced by the higher dose vaccine should lead to increased sterilizing immunity, yet this might still be lower than that expected in healthy young adults receiving the standard dose vaccine. Recently, a high-dose influenza vaccine (Fluzone® High-dose) was approved for use in elderly adults. Currently, the ACIP does not favor vaccination with the high-dose over the standard dose vaccine [54] and the level of protection of the high dose in comparison to the standard dose vaccine has yet to be determined. Adjuvanted inactivated influenza vaccines, some of which have been licensed in Europe since 1997, may provide superior protection. Annual vaccination with either the standard dose or high dose vaccine, with or without adjuvant, remains of utmost importance for individuals who may indeed benefit from vaccination; it maintains herd immunity and reduces the chances of infection of susceptible (perhaps unprotected) groups.

## Acknowledgments

The authors thank Yu Lim and Mardi Reymann, CVD Applied Immunology Section, for their outstanding technical support and Steven Bowen and Melissa Hayes, Department of Microbiology and Immunology, for their contribution to this work. We also acknowledge Dr. Linda Lambert, Chief of the Respiratory Diseases Branch at DMID, NIAID. This work was funded by NIH, NCRR grant K12-RR023250 (WHC), NIA grant P30-AG028747 (WHC), and NIAID contracts N01-AI85342, N01-AI25461 and N01-AI-800001 (WHC, AC, RE, MBS, MFP). ClinicalTrials.gov identifier: NCT00115531 and NCT00170508.

## Reference List


1. Thompson WW, Shay DK, Weintraub E, et al. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA. 2003 Jan 8; 289(2):179–86. [PubMed: 12517228]
2. Nichol KL, Nordin JD, Nelson DB, Mullooly JP, Hak E. Effectiveness of influenza vaccine in the community-dwelling elderly. N Engl J Med. 2007 Oct 4; 357(14):1373–81. [PubMed: 17914038]
3. Rivetti D, Jefferson T, Thomas R, et al. Vaccines for preventing influenza in the elderly. Cochrane Database Syst Rev. 2006; 3:CD004876. [PubMed: 16856068]
4. Jefferson T, Rivetti D, Rivetti A, Rudin M, Di PC, Demicheli V. Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review. Lancet. 2005 Oct 1; 366(9492):1165–74. [PubMed: 16198765]
5. Gross PA, Hermogenes AW, Sacks HS, Lau J, Levandowski RA. The efficacy of influenza vaccine in elderly persons. A meta-analysis and review of the literature. Ann Intern Med. 1995 Oct 1; 123(7):518–27. [PubMed: 7661497]
6. Vu T, Farish S, Jenkins M, Kelly H. A meta-analysis of effectiveness of influenza vaccine in persons aged 65 years and over living in the community. Vaccine. 2002 Mar 15; 20(13–14):1831–6. [PubMed: 11906772]
7. Chen WH, Kozlovsky BF, Effros RB, Grubeck-Loebenstein B, Edelman R, Sztein MB. Vaccination in the elderly: an immunological perspective. Trends Immunol. 2009 Jul; 30(7):351–9. [PubMed: 19540808]
8. Potter CW, Oxford JS. Determinants of immunity to influenza infection in man. Br Med Bull. 1979 Jan; 35(1):69–75. [PubMed: 367490]
9. Beyer WE, Palache AM, Baljet M, Masurel N. Antibody induction by influenza vaccines in the elderly: a review of the literature. Vaccine. 1989 Oct; 7(5):385–94. [PubMed: 2683459]
10. Goodwin K, Viboud C, Simonsen L. Antibody response to influenza vaccination in the elderly: a quantitative review. Vaccine. 2006 Feb 20; 24(8):1159–69. [PubMed: 16213065]
11. McElhaney JE, Upshaw CM, Hooton JW, Lechelt KE, Meneilly GS. Responses to influenza vaccination in different T-cell subsets: a comparison of healthy young and older adults. Vaccine. 1998 Nov; 16(18):1742–7. [PubMed: 9778750]
12. Ruben FL, Jackson GG. A new subunit influenza vaccine: acceptability compared with standard vaccines and effect of dose on antigenicity. J Infect Dis. 1972 Jun; 125(6):656–64. [PubMed: 4556572]
13. Mostow SR, Schoenbaum SC, Dowdle WR, Coleman MT, Kaye HS. Inactivated vaccines. 1. Volunteer studies with very high doses of influenza vaccine purified by zonal ultracentrifugation. Postgrad Med J. 1973 Mar; 49(569):152–8. [PubMed: 4802501]
14. Matzkin H, Nili E. Accidental tenfold overdose of influenza vaccine: a clinical and serological study. Isr J Med Sci. 1984 May; 20(5):411–5. [PubMed: 6469561]
15. Gross PA, Quinnan GV Jr, Weksler ME, Gaerlan PF, Denning CR. Immunization of elderly people with high doses of influenza vaccine. J Am Geriatr Soc. 1988 Mar; 36(3):209–12. [PubMed: 3339228]
16. Palache AM, Beyer WE, Sprenger MJ, et al. Antibody response after influenza immunization with various vaccine doses: a double-blind, placebo-controlled, multi-centre, dose-response study in elderly nursing-home residents and young volunteers. Vaccine. 1993; 11(1):3–9. [PubMed: 8427034]
17. Remarque EJ, van Beek WC, Ligthart GJ, et al. Improvement of the immunoglobulin subclass response to influenza vaccine in elderly nursing-home residents by the use of high-dose vaccines. Vaccine. 1993; 11(6):649–54. [PubMed: 8322488]
18. Keitel WA, Couch RB, Cate TR, et al. High doses of purified influenza A virus hemagglutinin significantly augment serum and nasal secretion antibody responses in healthy young adults. J Clin Microbiol. 1994 Oct; 32(10):2468–73. [PubMed: 7814484]
19. Keitel WA, Cate TR, Atmar RL, et al. Increasing doses of purified influenza virus hemagglutinin and subvirion vaccines enhance antibody responses in the elderly. Clin Diagn Lab Immunol. 1996 Sep; 3(5):507–10. [PubMed: 8877126]

20. Couch RB, Winokur P, Brady R, et al. Safety and immunogenicity of a high dosage trivalent influenza vaccine among elderly subjects. Vaccine. 2007 Nov 1; 25(44):7656–63. [PubMed: 17913310]
21. Couch RB, Winokur P, Brady R, et al. Safety and immunogenicity of a high dosage trivalent influenza vaccine among elderly subjects. Vaccine. 2007 Nov 1; 25(44):7656–63. [PubMed: 17913310]
22. Couch RB, Winokur P, Brady R, et al. Safety and immunogenicity of a high dosage trivalent influenza vaccine among elderly subjects. Vaccine. 2007 Nov 1; 25(44):7656–63. [PubMed: 17913310]
23. Sztein MB, Wasserman SS, Tacket CO, et al. Cytokine production patterns and lymphoproliferative responses in volunteers orally immunized with attenuated vaccine strains of Salmonella typhi. J Infect Dis. 1994 Dec; 170(6):1508–17. [PubMed: 7995991]
24. WHO Department of Communicable Disease Surveillance and Response. Global Influenza Programme. 2002. WHO Manual on Animal Influenza Diagnosis and Surveillance. Report No.: WHO/CDS/CSRN/NCS/2002.5 Rev.1
25. U.S. Department of Heath EaWPHSCfDC. The hemagglutination inhibition test for influenza viruses. Atlanta, GA: WHO Collaborative Center for Influenza. Biological Products Division; 1975. Ref Type: Serial (Book, Monograph)
26. Shortridge KF, Lansdell A. Serum inhibitors of A 2 -Hong Kong influenza virus haemagglutination. Microbios. 1972 Dec; 6(24):213–9. [PubMed: 4348986]
27. Capozzo AV, Ramirez K, Polo JM, et al. Neonatal immunization with a Sindbis virus-DNA measles vaccine induces adult-like neutralizing antibodies and cell-mediated immunity in the presence of maternal antibodies. J Immunol. 2006 May 1; 176(9):5671–81. [PubMed: 16622037]
28. Salerno-Goncalves R, Pasetti MF, Sztein MB. Characterization of CD8(+) effector T cell responses in volunteers immunized with Salmonella enterica serovar Typhi strain Ty21a typhoid vaccine. J Immunol. 2002 Aug 15; 169(4):2196–203. [PubMed: 12165550]
29. Salerno-Goncalves R, Wahid R, Sztein MB. Immunization of volunteers with Salmonella enterica serovar Typhi strain Ty21a elicits the oligoclonal expansion of CD8+ T cells with predominant Vbeta repertoires. Infect Immun. 2005 Jun; 73(6):3521–30. [PubMed: 15908381]
30. Chen WH, Kozlovsky BF, Effros RB, Grubeck-Loebenstein B, Edelman R, Sztein MB. Vaccination in the elderly: an immunological perspective. Trends Immunol. 2009 Jul; 30(7):351–9. [PubMed: 19540808]
31. Siegrist CA, Aspinall R. B-cell responses to vaccination at the extremes of age. Nat Rev Immunol. 2009 Mar; 9(3):185–94. [PubMed: 19240757]
32. Couch RB, Winokur P, Brady R, et al. Safety and immunogenicity of a high dosage trivalent influenza vaccine among elderly subjects. Vaccine. 2007 Nov 1; 25(44):7656–63. [PubMed: 17913310]
33. Remarque EJ, Cools HJ, Boere TJ, van der Klis RJ, Masurel N, Ligthart GJ. Functional disability and antibody response to influenza vaccine in elderly patients in a Dutch nursing home. BMJ. 1996 Apr 20.312(7037):1015. [PubMed: 8616350]
34. Palache AM, Beyer WE, Luchters G, Volker R, Sprenger MJ, Masurel N. Influenza vaccines: the effect of vaccine dose on antibody response in primed populations during the ongoing interpandemic period. A review of the literature. Vaccine. 1993; 11(9):892–908. [PubMed: 8212834]
35. Sullivan KM, Monto AS, Foster DA. Antibody response to inactivated influenza vaccines of various antigenic concentrations. J Infect Dis. 1990 Feb; 161(2):333–5. [PubMed: 2299213]
36. Treanor JJ, Schiff GM, Couch RB, et al. Dose-related safety and immunogenicity of a trivalent baculovirus-expressed influenza-virus hemagglutinin vaccine in elderly adults. J Infect Dis. 2006 May 1; 193(9):1223–8. [PubMed: 16586358]
37. Remarque EJ, de Bruijn IA, Boersma WJ, Masurel N, Ligthart GJ. Altered antibody response to influenza H1N1 vaccine in healthy elderly people as determined by HI, ELISA, and neutralization assay. J Med Virol. 1998 May; 55(1):82–7. [PubMed: 9580890]

38. McElhaney JE, Meneilly GS, Lechelt KE, Beattie BL, Bleackley RC. Antibody response to whole-virus and split-virus influenza vaccines in successful ageing. Vaccine. 1993; 11(10):1055–60. [PubMed: 8212827]
39. Pyhala R, Kinnunen L, Kumpulainen V, Ikonen N, Kleemola M, Cantell K. Vaccination-induced HI antibody to influenza A(H1N1) viruses in poorly primed adults under circumstances of low antigenic drift. Vaccine. 1993; 11(10):1013–7. [PubMed: 8212820]
40. Couch RB, Winokur P, Brady R, et al. Safety and immunogenicity of a high dosage trivalent influenza vaccine among elderly subjects. Vaccine. 2007 Nov 1; 25(44):7656–63. [PubMed: 17913310]
41. Couch RB, Winokur P, Brady R, et al. Safety and immunogenicity of a high dosage trivalent influenza vaccine among elderly subjects. Vaccine. 2007 Nov 1; 25(44):7656–63. [PubMed: 17913310]
42. Falsey AR, Treanor JJ, Tornieporth N, Capellan J, Gorse GJ. Randomized, double-blind controlled phase 3 trial comparing the immunogenicity of high-dose and standard-dose influenza vaccine in adults 65 years of age and older. J Infect Dis. 2009 Jul 15; 200(2):172–80. [PubMed: 19508159]
43. Keitel WA, Cate TR, Atmar RL, et al. Increasing doses of purified influenza virus hemagglutinin and subvirion vaccines enhance antibody responses in the elderly. Clin Diagn Lab Immunol. 1996 Sep; 3(5):507–10. [PubMed: 8877126]
44. Keitel WA, Atmar RL, Cate TR, et al. Safety of high doses of influenza vaccine and effect on antibody responses in elderly persons. Arch Intern Med. 2006 May 22; 166(10):1121–7. [PubMed: 16717175]
45. Powers DC, Kilbourne ED, Johansson BE. Neuraminidase-specific antibody responses to inactivated influenza virus vaccine in young and elderly adults. Clin Diagn Lab Immunol. 1996 Sep; 3(5):511–6. [PubMed: 8877127]
46. Powers DC. Effect of age on serum immunoglobulin G subclass antibody responses to inactivated influenza virus vaccine. J Med Virol. 1994 May; 43(1):57–61. [PubMed: 8083650]
47. de Bruijn IA, Remarque EJ, Jol-van der Zijde CM, van Tol MJ, Westendorp RG, Knook DL. Quality and quantity of the humoral immune response in healthy elderly and young subjects after annually repeated influenza vaccination. J Infect Dis. 1999 Jan; 179(1):31–6. [PubMed: 9841819]
48. El-Madhun AS, Cox RJ, Haaheim LR. The effect of age and natural priming on the IgG and IgA subclass responses after parenteral influenza vaccination. J Infect Dis. 1999 Oct; 180(4):1356–60. [PubMed: 10479172]
49. Brokstad KA, Cox RJ, Major D, Wood JM, Haaheim LR. Cross-reaction but no avidity change of the serum antibody response after influenza vaccination. Vaccine. 1995 Nov; 13(16):1522–8. [PubMed: 8578836]
50. Dong L, Mori I, Hossain MJ, Kimura Y. The senescence-accelerated mouse shows aging-related defects in cellular but not humoral immunity against influenza virus infection. J Infect Dis. 2000 Aug; 182(2):391–6. [PubMed: 10915067]
51. Scherle PA, Gerhard W. Differential ability of B cells specific for external vs. internal influenza virus proteins to respond to help from influenza virus-specific T-cell clones in vivo. Proc Natl Acad Sci U S A. 1988 Jun; 85(12):4446–50. [PubMed: 3260034]
52. Palladino G, Scherle PA, Gerhard W. Activity of CD4+ T-cell clones of type 1 and type 2 in generation of influenza virus-specific cytotoxic responses in vitro. J Virol. 1991 Nov; 65(11): 6071–6. [PubMed: 1920626]
53. Murasko DM, Bernstein ED, Gardner EM, et al. Role of humoral and cell-mediated immunity in protection from influenza disease after immunization of healthy elderly. Exp Gerontol. 2002 Jan; 37(2–3):427–39. [PubMed: 11772530]
54. Advisory Committee on Immunization Practices (ACIP). Provisional Recommendations for the Use of Influenza Vaccines, vote February 24, 2010. CDC; 10 A.D March 2.

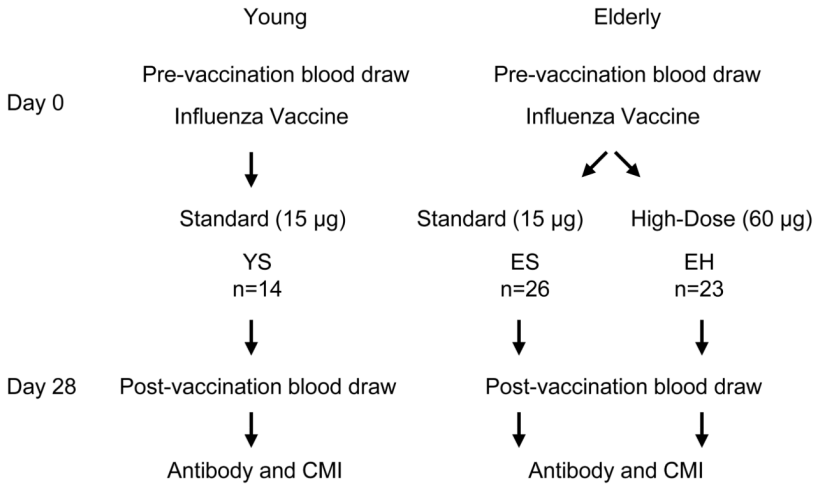


**Figure 1.**
Flowchart outlining the study. YS, young standard-dose vaccine; ES, elderly standard-dose vaccine; EH, elderly high-dose vaccine; CMI, cell-mediated immunity.

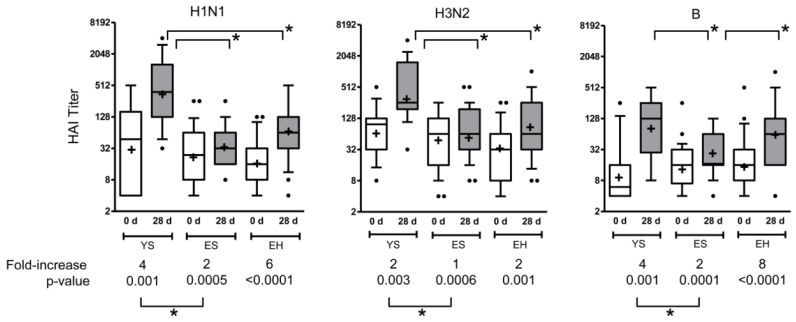


**Figure 2.**

Antibody responses to influenza vaccine antigens measured by hemagglutination inhibition (HAI) in elderly and young adults. Data shown are the median HAI titers and 25–75 percentile (boxes), 10–90 percentile (whiskers), geometric mean titers (GMT,+), and outliers (dots) for the 3 strains contained in the vaccine, measured for each cohort on days 0 and 28; shaded boxes represent post-vaccination data. Median fold increase in post-vaccination titer and p value for comparing the median log-transformed fold-increase to 0 by two-sided Wilcoxon signed-rank test are indicated. Horizontal lines indicate groups with a significant difference in post-vaccination GMT (brackets on top) or median log-transformed fold-increase (brackets on bottom). In ANOVA for log-transformed day 28 titer, $p < 0.0001$ for H1N1, 0.004 for H3N2, 0.008 for B. In Kruskal-Wallis ANOVA for log-transformed fold-increase, $p = 0.003$ for H1N1, 0.026 for H3N2, 0.0004 for B. * denotes $p<0.05$ for pairwise comparison between groups, based on the Tukey-Kramer multiple comparisons procedure (log of day 28 titer) or the Dunn multiple comparisons procedure for ranks (log of fold-increase).

**Figure 3.**

Antibody responses to influenza vaccine antigens measured by virus neutralization (VN). Data shown are the median VN titers and 25–75 percentile (boxes), 10–90 percentile (whiskers), geometric mean titers (GMT,+), and outliers (dots) for the 3 strains contained in the vaccine, measured for each cohort on days 0 and 28; shaded boxes represent post-vaccination data. Median fold-increase in post-vaccination titer and p value for comparing the median log-transformed fold-increase to 0 by two-sided Wilcoxon signed-rank test are indicated. Horizontal lines indicate groups with a significant difference in post-vaccination GMT (brackets on top) or median log-transformed fold-increase (brackets on bottom). In ANOVA for log-transformed day 28 titer, p = 0.004 for H1N1, 0.004 for H3N2, 0.058 for B). In Kruskal-Wallis ANOVA for log-transformed fold-increase, p = 0.007 for H1N1, 0.003 for H3N2, 0.002 for B. * denotes $p<0.05$ for pairwise comparison between groups, based on the Tukey-Kramer multiple comparisons procedure (log of day 28 titer) or the Dunn multiple comparisons procedure for ranks (log of fold increase).

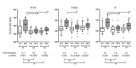

**Figure 4.**

IgG antibody responses to influenza vaccine antigens measured by ELISA. Data shown are the median virus-specific serum IgG titers and 25–75 percentile (boxes), 10–90 percentile (whiskers), geometric mean titers (GMT,+), and outliers (dots) for the 3 strains contained in the vaccine, measured for each cohort on days 0 and 28; shaded boxes represent post-vaccination data. Median fold-increase in post-vaccination titer and p value for comparing the median log-transformed fold-increase to 0 by two-sided Wilcoxon signed-rank test are indicated. Horizontal lines indicate groups with a significant difference in post-vaccination GMT (brackets on top) or median log-transformed fold-increase (brackets on bottom). In ANOVA for log-transformed day 28 titer, $p<0.0001$ for H1N1, $p=0.003$ for H3N2, $p<0.0001$ for B. In Kruskal-Wallis ANOVA for log-transformed fold-increase, $p=0.002$ for H1N1, $p=0.006$ for H3N2, $p=0.0004$ for B. * denotes $p<0.05$ for pairwise comparison between groups, based on the Tukey-Kramer multiple comparisons procedure (log of day 28 titer) or the Dunn multiple comparisons procedure for ranks (log of fold-increase).

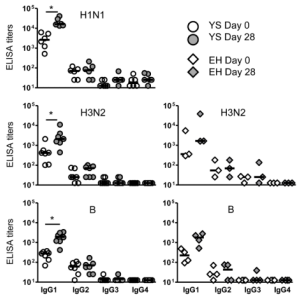


**Figure 5.**

Virus-specific IgG subclasses in vaccine responders. Scatter plots show individual IgG1, IgG2, IgG3 and IgG4 titers before and after vaccination and median responses (line) for the YS and EH cohorts. There were no ELISA IgG responders in the ES cohort for any of the three virus strains or for the EH cohort with H1N1 (not shown). * denotes $p<0.05$ for comparing titers before and after vaccination by two-sided Wilcoxon signed-rank test. ($p=0.036$ for H1N1, 0.022 for H3N2, 0.022 for B)

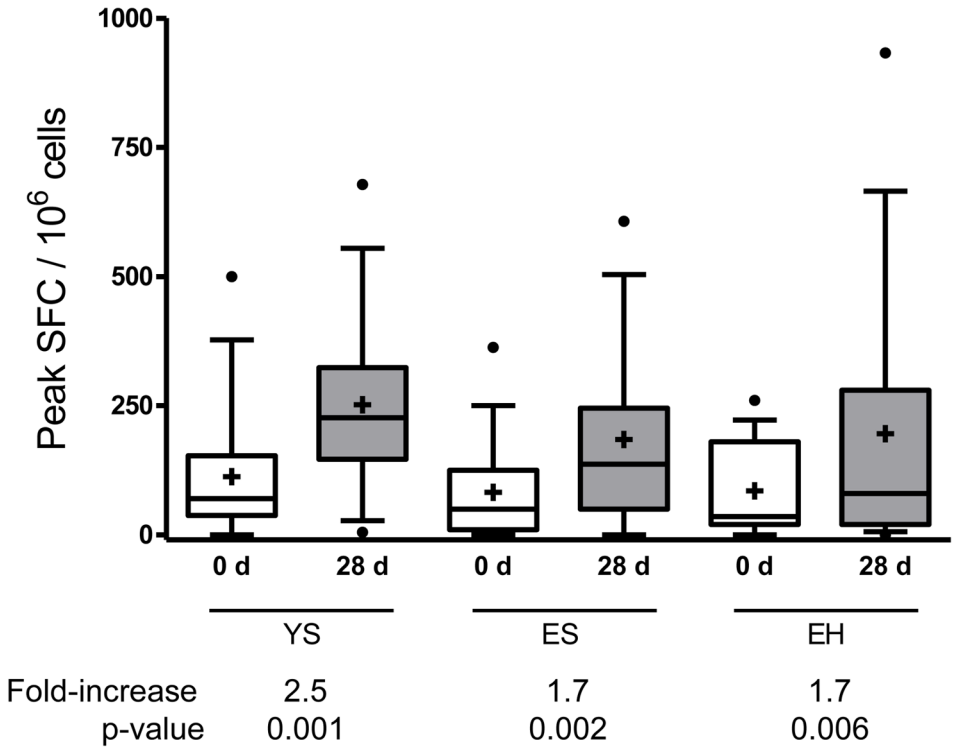


**Figure 6.**

IFN-γ responses to influenza vaccination in elderly and young adults. Data show the median net frequency of IFN-γ spot forming cells (SFC) per $10^6$ PBMC and 25–75 percentile (boxes), 10–90 percentile (whiskers), mean (+), and outliers (dots). Median fold-increase in IFN-γ SFC after vaccination is indicated; p values were calculated comparing pre- vs. post-vaccination SFC responses by two-sided Wilcoxon signed-rank test.

## Table 1

Demographic characteristics of the vaccine recipients

| | Subjects/Influenza Vaccine Dose | | |
|---|---|---|---|
| | **Young/Standard** | **Elderly/Standard** | **Elderly/High-Dose** |
| N | 14 | 26 | 23 |
| Age (mean±SD) | 28±6 | 74±6 | 74±5 |
| Sex (% male) | 64% | 50% | 52% |
| Race (% white) | 71% | 100% | 100% |
| Flu vaccine previous year | 29% | 81% | 78% |

## Table 2

Antibody responses to influenza vaccine antigens measured by hemagglutination inhibition (HAI) in elderly and young adults. (A) Percent of subjects that demonstrated “seroprotection” (≥1:32) or “seroconversion” (4-fold rise) in strain-specific responses. (B) Percent of all subjects or only subjects that received influenza vaccination the prior season that demonstrated a “seroprotective” (≥1:32) response to 1, 2, or all 3 of the vaccine-strains.

**A.**

| | | N | HAI titers ≥1:32 | 4-fold rise |
|---|---|---|---|---|
| H1N1 | YS | 14 | 100 | 64 |
| | ES | 26 | 62 | 15 |
| | EH | 23 | 83 | 52 |
| H3N2 | YS | 14 | 100 | 43 |
| | ES | 26 | 85 | 11 |
| | EH | 23 | 87 | 39 |
| B | YS | 14 | 79 | 79 |
| | ES | 26 | 46 | 19 |
| | EH | 23 | 74 | 61 |

**B.**

| | | | Subjects with HAI titers ≥1:32 | | |
|---|---|---|---|---|---|
| | | N | 1 | 2 | 3 |
| All participants | YS | 14 | 100 | 100 | 79 |
| | ES | 26 | 100 | 65 | 27 |
| | EH | 23 | 91 | 87 | 65 |
| Prior Flu Immunization | YS | 4 | 100 | 100 | 50 |
| | ES | 21 | 100 | 71 | 33 |
| | EH | 18 | 89 | 83 | 61 |