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Antibody-Dependent Cellular Cytotoxicity Responses to Seasonal Influenza Vaccination in Older Adults

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Background. Older adults are at high risk of influenza disease, but generally respond poorly to vaccination. Antibody-dependent cellular cytotoxicity (ADCC) may be an important component of protection against influenza infection. An improved understanding of the ADCC response to influenza vaccination in older adults is required.

Methods. We studied sera samples from 3 groups of subjects aged ≥65 years (n = 16–17/group) receiving the 2008/2009 seasonal trivalent influenza vaccine (TIV). Subjects had minimal pre-existing hemagglutination inhibiting (HAI) antibodies and TIV induced either no, low, or high HAI responses. Serum ADCC activity was analyzed using Fc receptor cross-linking, NK cell activation, and influenza-infected cell killing.

Results. Most subjects from TIV nonresponder, low responder, and high responder groups had detectable ADCC antibodies prevaccination, but baseline ADCC was not predictive of HAI vaccine responsiveness. Interestingly, ADCC and HAI responses tracked closely across all groups, against all 3 TIV hemagglutinins, and in all ADCC assays tested.

Conclusions. Older adults commonly have pre-existing ADCC antibodies in the absence of high HAI titers to circulating influenza strains. In older vaccinees, ADCC response mirrored HAI antibodies and was readily detectable despite high postvaccination HAI titers. Alternate measures of vaccine responsiveness and improved vaccinations in this at-risk group are needed.

Keywords. influenza; vaccine; older adults; ADCC.

Older adults bear the greatest burden of influenza-related disease [1]. Comorbidities and immune senescence increase their susceptibility to influenza-related pneumonia and other complications, hospitalization, and death [1]. Vaccination is an effective method of reducing influenza infection in older adults [2–4] and is widely recommended [5]. The seasonal influenza vaccine is updated and administered annually [5], but is only modestly protective when circulating and selected influenza vaccine strains are mismatched [6].

Antibodies are important for influenza protection [7]. Inactivated split-virion vaccines can induce neutralizing antibodies (NAbs) [8] that inhibit influenza entry into host cells and prevent infection [9]. However, the immune response to influenza vaccination declines with age [10–12]. Older adults have decreased generation of memory B cells, long-lived plasma cells, and serum antibodies [13, 14]. High dose and adjuvanted influenza vaccines improve efficacy in older adults [15–18], but increase reactogenicity [16]. Murasko et al showed that older adults demonstrate significant heterogeneity in their ability to generate humoral and cellular immune responses to seasonal influenza vaccination [12]. Reduced activation-induced cytidine deaminase activity in B cells and elevated $CD8^+CD28^-$ T cells are associated with impaired responses to influenza vaccination [13, 14, 19], but other factors are poorly understood.

Hemagglutination inhibition (HAI) assays detect influenza antibodies that bind strain-specific epitopes adjacent to the receptor binding site of hemagglutinin (HA) and block viral entry. The HAI assay has been an important surrogate of vaccine-induced immunity against influenza for decades, but antibody-dependent cellular cytotoxicity (ADCC) is increasingly recognized as another potential mediator of influenza immunity [20–31]. ADCC is induced when Fc receptors on the surface of innate effector cells engage the Fc region of IgG bound to viral antigens on infected cells. Cross-linking FcγRs on the effector cell causes activation and release of antiviral cytokines and cytolytic granules. ADCC is primarily mediated through human FcγRIIIa found on natural killer (NK) cells, monocytes, and macrophages [32]. Antibodies with ADCC activity can be broadly cross-reactive and commonly target more conserved epitopes, which is desirable for protection against diverse subtypes and vaccine mismatched strains of influenza [20, 21]. ADCC antibodies are induced by seasonal influenza

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vaccination in healthy children and adults [29, 33, 34], but have not previously been examined in older adults.

Herein, sera from 3 groups of subjects aged ≥65 years, who responded differently to the standard trivalent influenza vaccine (TIV) by HAI, were assessed to determine whether influenza-specific ADCC mirrored HAI antibodies or revealed differences that may be important in dissecting influenza immunity. Recent studies performed with monoclonal antibodies (mAbs) and polyclonal sera from H5N1 vaccinated subjects have shown that HAI antibodies are inhibitory to ADCC [25, 31, 35], thus we expect ADCC activity to be reduced in older adults who demonstrate postvaccination increases in HAI antibodies.

MATERIALS AND METHODS

Influenza Vaccination Cohort

A cohort of 1249 subjects aged ≥65 years completed a TIV clinical trial in 2008 in the United States (https://clinicaltrials.gov #NCT00735475) sponsored by Seqirus Ltd. Subjects received the 2008/2009 TIV composed of inactivated A/Brisbane/59/2007(H1N1) virus, A/Uruguay/716/2007(H3N2) virus, and B/Florida/04/2006 virus. Prevaccine (day 0) and postvaccine (day 21) blood samples were obtained. HAI assays were performed as previously described [34].

Selection of Influenza Vaccination Cohort for ADCC Studies Based on HAI Titer

We aimed to study ADCC in 50 subjects with a range of HAI responses to vaccination. Participants were divided into responder and nonresponder groups based on their seroconversion to at least 2 out of 3 TIV viruses at day 21 postvaccination. Seroconversion was defined as ≥4-fold rise in HAI geometric mean titer (GMT) and HAI GMT of ≥40 postvaccination, with 692 subjects (55.4%) classified as nonresponders and 557 (44.6%) as responders. Responders were further divided into low responders and high responders based on the magnitude of their HAI GMT increase postvaccination. Subjects who demonstrated a greater than 20-fold increase in HAI GMT to at least 2 of the 3 TIV viruses postvaccination were classified as high responders. Responders who demonstrated a ≤20-fold increase in HAI GMT to at least 2 of the 3 TIV viruses were classified as low responders (Figure 1). To study responsiveness in subjects with minimal prevaccination immunity, the responder groups were down-selected based on low prevaccination HAI GMTs and the nonresponder group was selected for low HAI GMTs both pre- and postvaccination. Sera from a total of 50 subjects were tested for Fc-functional antibodies; this allowed (1) sufficient subjects to detect correlations with HAI responses [34, 36, 37] and (2) provided a technically manageable number of samples for assays. Based on these selection criteria we identified 16 nonresponders, 17 low responders, and 17 high responders to analyze intensively for serum ADCC-mediating antibodies (Figure 1 and Supplementary Table 1).

Hemagglutinin Proteins

Recombinant HA proteins were purchased from Sinobiological (Shanghai, China). Due to lack of availability, the antigenically similar HA protein of A/Brisbane/10/2007(H3N2) virus was used instead of A/Uruguay/716/2007(H3N2) HA to perform ADCC assays.

Dimeric Recombinant Soluble FcγRIIIa (CD16a) Binding ELISA

A recombinant soluble FcγRIIIa (rsFcγRIIIa) dimer enzyme-linked immunosorbent assay (ELISA) was used to model the need for ADCC-mediating antibodies to cross-link FcγRs. The rsFcγRIIIa dimer was provided by Dr B. Wines (Burnet Institute, Melbourne, Australia), produced as previously described [37]. Briefly, 96-well ELISA plates were coated with 50 ng of purified influenza HA or HIV-1 gp140 control protein overnight. Sera dilutions were added, incubated for 1 hour, washed, and 50 µL of 0.1 µg/mL biotinylated rsFcγRIIIa dimer (V176 high affinity variant) added for 1 hour. Streptavidin- horseradish peroxidase (HRP) (1:10 000, ThermoFisher Scientific, Waltham, MA) was added for 1 hour, washed, blotted dry, then 50 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma Aldrich, St Louis, MO) was added and the plate was developed. The reaction was stopped and absorbance read at 450 nm. Intragam 5 (5 µg/mL, bioCSL, Melbourne, Australia) was used as a positive control and allowed for normalization between plates.

Antibody-Mediated NK Cell Activation

Natural killer cell activation measured the capacity of antibodies bound to a plate coated with immobilized influenza proteins to induce NK-92-FcγRIIIa-GFP cell expression of CD107a, as previously described [34, 36]. Briefly, 96-well ELISA plates were coated with 600 ng of influenza HA or HIV-1 gp140 protein, then incubated with a 1:40 dilution of sera for 2 hours at 37°C. Then 2×10^5 NK-92-FcγRIIIa-GFP cells (expressing the V176 variant of FcγRIIIa conjugated to green fluorescent protein [GFP], provided by Dr K. Campbell, Institute for Cancer Research. Philadelphia, PA) were added for 5 hours. The NK-92-FcγRIIIa-GFP cells were then incubated with 1 mM ethylenediaminetetraacetic acid (EDTA) and anti-CD107a allophycocyanin antibody (clone H4A3; BD Biosciences, San Jose, CA) for 30 minutes. Cells were washed, fixed with formaldehyde, and acquired on a flow cytometer. The proportion of NK-92-FcγRIIIa-GFP$^+$ cells expressing CD107a was analyzed.

ADCC Killing of Influenza-Infected Cells

A lactate dehydrogenase (LDH) release ADCC assay was used as previously described [38, 39]. Briefly, A549 respiratory cell targets were infected with either A/Brisbane/59/2007(H1N1) or B/Florida/04/2006 influenza viruses at a multiplicity of infection (MOI) of 5 or 10, respectively, for 5 hours. Experimental wells contained 2×10^4 NK-92-FcγRIIIa-GFP cells combined with 2×10^4 influenza-infected A549 cells in an effector:target ratio of 1:1 and a dilution of heat-inactivated plasma from vaccinated

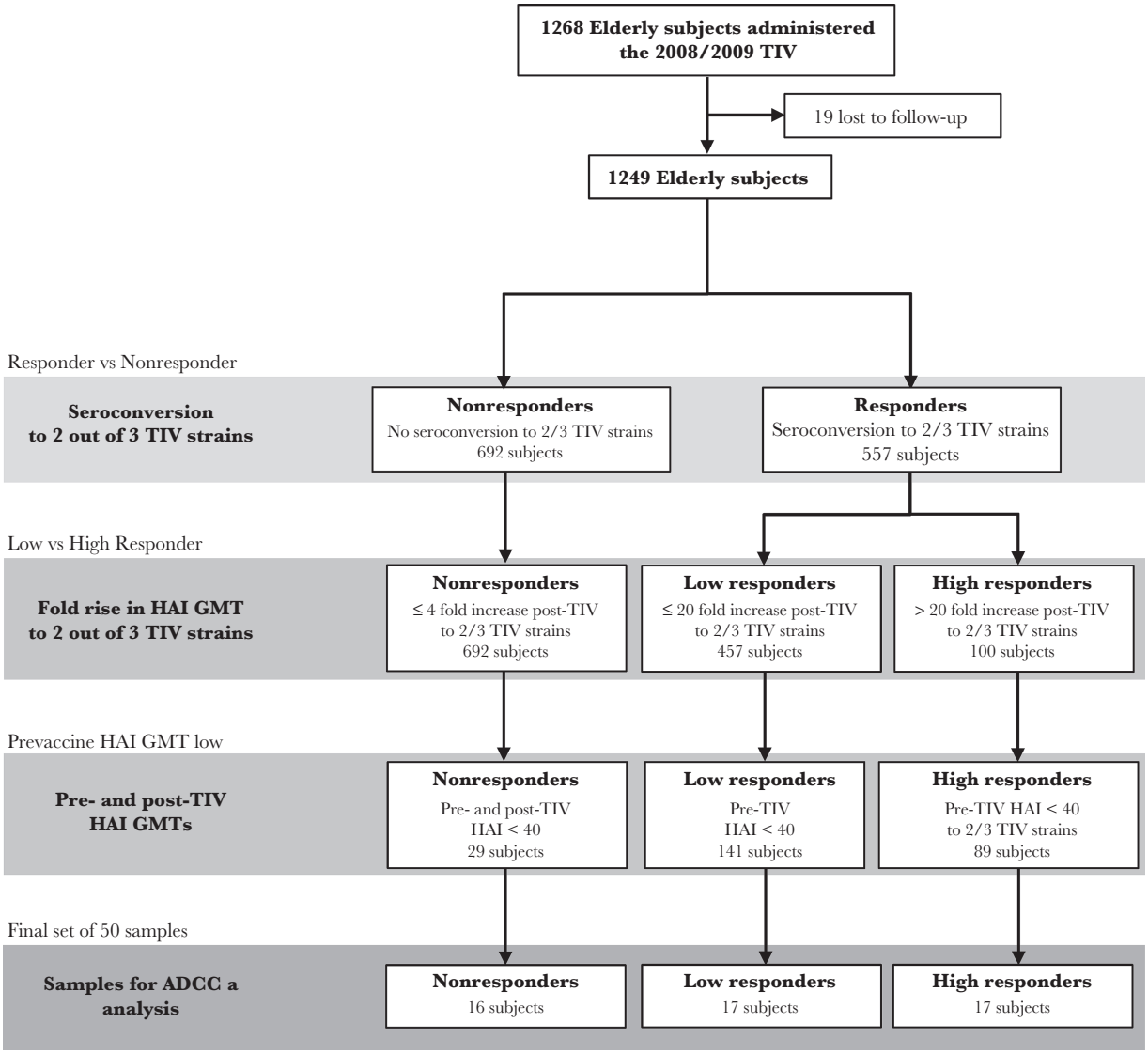

Figure 1. Flow diagram depicting selection of 50 older adults from a 2008/2009 seasonal influenza vaccine clinical trial for serum antibody-dependent cellular cytotoxicity (ADCC) analysis. Older adults who completed a 2008/2009 seasonal influenza vaccine clinical trial (n = 1249) were divided into vaccine nonresponders and responders based upon seroconversion to at least 2 out of 3 vaccine strains day 21 postvaccination. Vaccine responders were further subdivided into low responders and high responders depending on the magnitude of their postvaccination rise in hemagglutination inhibiting (HAI) geometric mean titers (GMTs) to at least 2 out of 3 vaccine strains as indicated. To study subjects without prior seroprotective HAI GMTs, we then identified subjects in these 3 groups with low baseline HAI GMTs. Finally, we selected a cohort of 50 subjects to analyze for ADCC antibodies from the nonresponder, low responder, and high responder groups. Abbreviation: TIV, trivalent influenza vaccine.

donors in triplicate (1:5000 dilution for A/Brisbane/59/2007-infected targets and 1:1000 for B/Florida/04/2006-infected targets). LDH release was measured with the Cytotox 96 kit (Promega, Madison, WI). Percent cytotoxicity was calculated: [(experimental – effector spontaneous – target spontaneous)/(maximum LDH – target spontaneous)]. Minimal killing is detected in the absence of antibody (<0.2% killing) or against uninfected A549 cells (<0.8% killing) for this LDH assay.

IgG Subclass Multiplex

The IgG subclass multiplex was performed as previously described for HIV [40]. Bio-Plex Pro Magnetic COOH beads (BioRad, Hercules, CA) were coupled to the 3 influenza HA proteins or HIV gp140 protein, using 1.25 million beads per antigen, each with different fluorescent properties. A working microsphere mix containing 1000 of each microsphere type/well was combined with a 1:100 dilution of donor sera into the wells of a black, clear bottom 96-well plate (BioRad). Anti-human IgG1-4 antibodies conjugated to R-phycoerythrin (PE 1.3 μg/mL; SouthernBiotech, Birmingham, AL) were added to detect antigen-specific IgG bound to fluorescent beads. The plate was washed using the Bio-Plex Pro II Wash Station and read on the BioRad Bio-Plex MAGPIX Multiplex reader (BioRad). Binding of the PE detector antibodies was measured to calculate median fluorescence intensity (MFI).

Principal Component Analysis

Principal component analysis (PCA) [41] method was used to cluster and reduce the dimension of the outcome variables as previously described [42]. Analysis was performed using Matlab with Statistics and Machine Learning Toolbox.

RESULTS

Pre- and Postvaccination HAI Titers in Older Adults

Hemagglutination inhibiting responses to influenza vaccination are highly heterogeneous in older adults, likely related in part to a variable and unknown history of prior influenza infection/vaccination. The relationship between baseline HAI titers and response to influenza vaccination in older adults is of interest. We selected a group of 50 subjects aged 65 years or older who lacked seroprotective HAI titers to most of the vaccine strains, but responded dichotomously to vaccination. We classified subjects as either nonresponders (no seroconversion and ≤4-fold rise to at least 2 of the 3 TIV strains), low responders (seroconversion and ≤20-fold rise to at least 2 of the 3 TIV strains), or high responders (seroconversion and >20-fold rise to at least 2 of the 3 TIV strains).

As a result of the selection criteria, the majority of the 50 subjects studied lacked seroprotective HAI GMTs (HAI<40) to A/Brisbane/59/2007 (H1N1, 50/50 subjects), A/Uruguay/716/2007 (H3N2, 43/50), and B/Florida/04/2006 (49/50) influenza strains prevaccination (Figure 2). Baseline median HAI titers were similar between the different nonresponder and responder groups (median HAI titer range 5–20 for all vaccine strains).

Because our older adult TIV cohort was divided based on HAI responsiveness, nearly all responders seroconverted (97% or 33/34) to the A/Brisbane/59/2007(H1N1) virus at day 21 postvaccination, while no nonresponders seroconverted. Median A/Brisbane/59/2007(H1N1) HAI titers postvaccination were significantly different between the groups (nonresponders = 10, low responders = 80, and high responders = 320; Figure 2A). Similarly, all 34 responders and none of the 16 nonresponders seroconverted to the A/Uruguay/716/2007(H3N2) virus post-TIV (median HAI titers nonresponders = 20, low responders = 320, and high responders = 1280; Figure 2B). The B/Florida/04/2006 virus elicited a lower HAI response than the H1N1 or H3N2 viruses in the 2008/2009 TIV. At day 21 postvaccination none of the nonresponders (0/16), 53% (9/17) of the low responders, and 94% (16/17) of the high responders seroconverted to the B/Florida/04/2006 virus. Postvaccination HAI titers to the B/Florida/04/2006 virus were significantly different between the nonresponder (median = 8.95), low responder (median = 40), and high responder (median = 160) groups (Figure 2C).

Postvaccination Rise in HA-Specific FcγRIIIa Cross-Linking Antibodies in Older Adult TIV Responders

Hemagglutinin-specific antibodies with Fc-mediated effector functions assist in the control and clearance of influenza virus

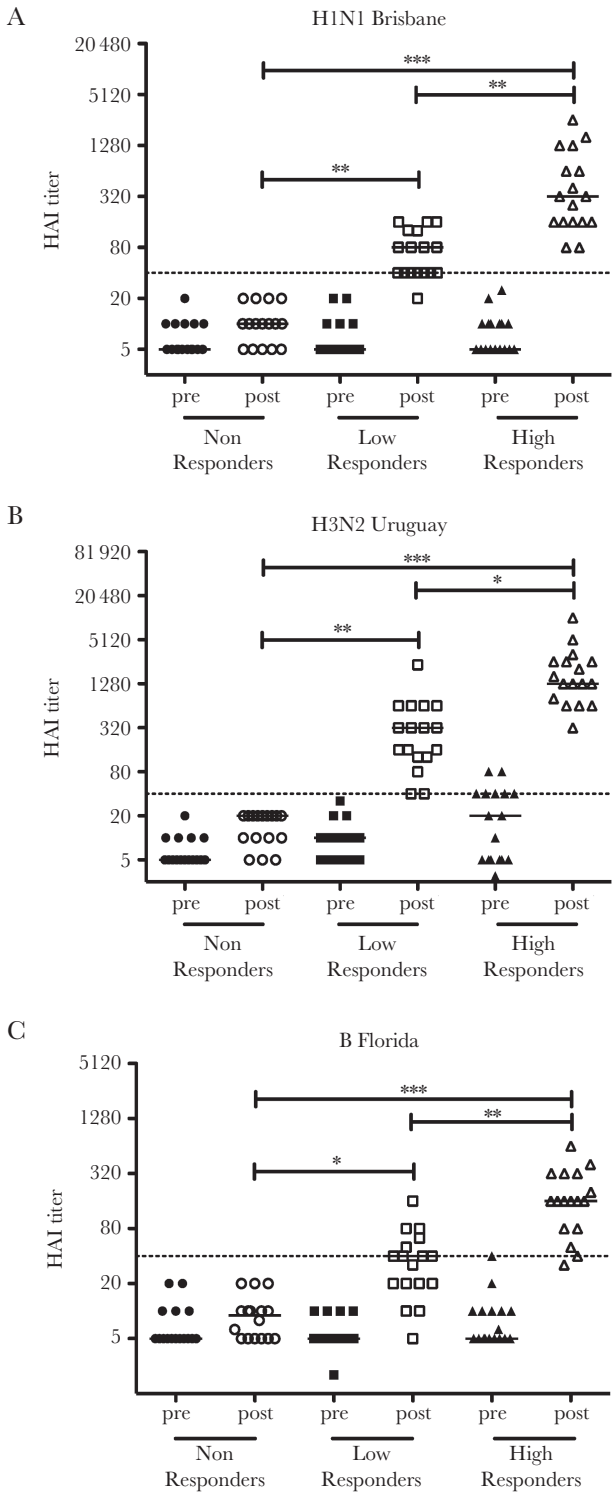

Figure 2. Pre- and postvaccination hemagglutination inhibiting (HAI) geometric mean titers (GMTs) to the 2008/2009 seasonal trivalent influenza vaccine (TIV) strains in older adults. Pre- (closed symbols) and postvaccine (open symbols) HAI GMTs against the A/Brisbane/59/2007(H1N1) virus (*A*), the A/Uruguay/716/2007(H3N2) virus (*B*), and the B/Florida/04/2006 virus (*C*) are shown for nonresponders (circles), low responders (squares), and high responders (triangles). Dotted lines denote an HAI GMT of 40. A Kruskal–Wallis test was performed with a Dunn's multiple comparison post hoc test. *$P < .05$, ** $P < .01$, ***$P < .001$.

infections in animal models [20, 21, 26, 31]. We first studied pre- and post-TIV sera from the 50 subjects using a recently developed Fc receptor dimer binding ELISA [37]. Participants classified as low or high responders by HAI demonstrated a significant rise in rsFcγRIIIa dimer binding antibodies post-vaccination against HA proteins from all 3 vaccine strains (Figure 3A–C). The nonresponder group, however, showed no significant increase in dimeric rsFcγRIIIa cross-linking

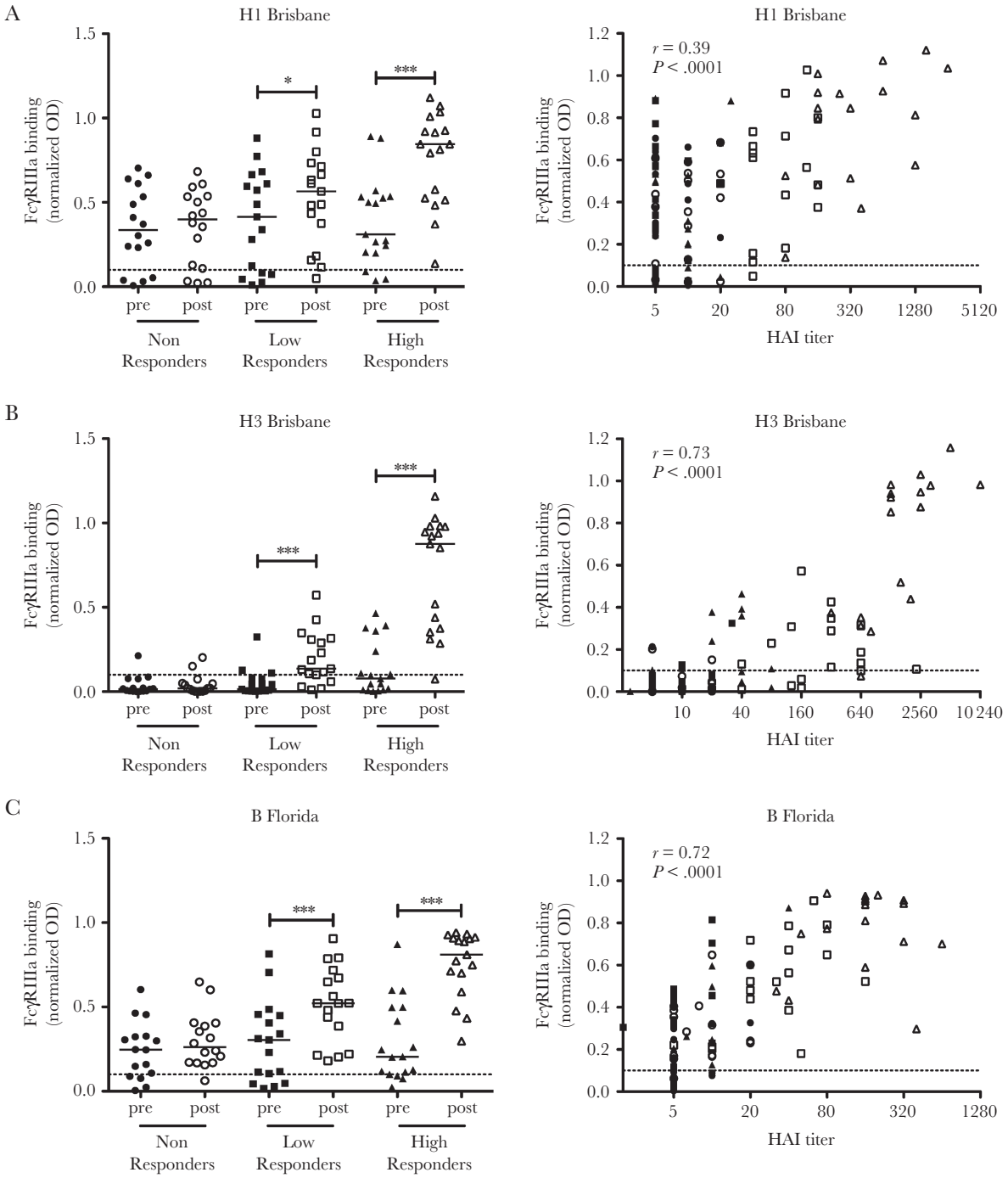

Figure 3. Increase in FcγRIIIa cross-linking antibodies against the hemagglutinins (HAs) of the 2008/2009 seasonal trivalent influenza vaccine (TIV) viruses in postvaccination sera from older adult responders. FcγRIIIa dimer binding antibodies against HA proteins from 3 TIV strains, A/Brisbane/59/2007(H1N1) virus (*A*), A/Brisbane/10/2007(H3N2) virus (*B*), and B/Florida/04/2006 virus (*C*), are shown in pre- (closed symbols) and postvaccination (open symbols) sera samples from older adult nonresponders (circles), low responders (squares), and high responders (triangles). Spearman correlations between serum FcγRIIIa cross-linking antibodies and HAI GMTs are also shown for the H1N1 (*A*), H3N2 (*B*), and B (*C*) TIV strains. All pre- and postvaccination sera samples were also tested for dimeric rsFcγRIIIa binding antibodies against an irrelevant HIV-1 protein gp140 and background was subtracted for each serum sample. Dotted lines denote a normalized optical density (OD) of 0.1, the limit of detection for the FcγRIIIa dimer ELISA. Wilcoxon-matched pairs signed rank tests were used to compare pre- and postvaccination samples. * $P < .05$, ** $P < .01$, *** $P < .001$.

antibodies against homologous influenza HAs (Figure 3A–C). Pre- and postvaccination sera samples from older adults demonstrated positive correlations between HA-specific rsFcγRIIIa dimer binding antibodies and HAI titers for all 3 vaccine strains (all P <.0001, Figure 3A–C). Subjects from all groups had readily detectable FcγRIIIa cross-linking antibodies against the HAs of A/Brisbane/59/2007(H1N1) and B/Florida/04/2006 viruses prevaccination (Figure 3A and C). This illustrates that older adults have cross-reactive HA-specific Fc-functional antibodies at baseline and a postvaccination rise in HAI GMT results in an associated increase of these Fc-functional antibodies.

Increased NK Cell Activation by HA-Specific Antibodies Postvaccination in Older Adult TIV Responders

Multimeric engagement of FcγRIIIa activates NK cells to express the degranulation marker CD107a. To confirm that the FcγRIIIa dimer ELISA results translate into functional NK cell activation, we tested samples for their ability to induce NK cell CD107a expression. High responder sera samples demonstrated increased antibody-dependent NK cell activation postvaccination against HA proteins from all 3 strains in the TIV (Figure 4A–C). Similarly, low responder sera samples showed greater NK cell activation postvaccination against the HAs of A/Brisbane/10/2007(H3N2) and B/Florida/04/2006 viruses. We also observed a trend towards greater NK cell activation post-TIV against the HA of A/Brisbane/59/2007(H1N1), but it did not reach statistical significance (P = .08). Consistent with the FcγRIIIa dimer binding data, there was no detectable difference in NK cell activation between pre- and postvaccination sera samples from nonresponders against the HAs of the 2 influenza A viruses tested (Figure 4A and B). Interestingly, a modest increase in NK cell activation was observed day 21 post-TIV against the HA of B/Florida/04/2006 in the nonresponder group (Figure 4C). A significant positive correlation was observed between antibody-dependent NK cell activation and HAI titers for all 3 TIV viruses (Figure 4A–C).

Cross-Reactivity of FcγRIIIa Dimer Binding Antibodies to Avian Influenza A Viruses Postvaccination

Vaccination of older adult TIV responders may expand broadly cross-reactive ADCC antibodies [21, 25]. The breadth of serum FcγRIIIa cross-linking antibodies was assessed against HAs from avian-origin H5N1 and H7N9 influenza viruses to which prior direct exposure was unlikely. ADCC antibodies against the HA of A/Vietnam/1194/2005(H5N1) were present in less than half of the subjects at baseline and were only significantly boosted in the high responder group (Figure 5A). FcγRIIIa cross-linking antibodies against the HA of A/Shanghai/1/2013(H7N9) virus were not detectable in nonresponder or low responder groups (Figure 5B). Five high responders did generate detectable dimeric FcγRIIIa binding antibodies against the HA of avian H7N9 virus postvaccination (Figure 5B). Interestingly, 3 of these 5 subjects also demonstrated a large (>5-fold) postvaccination increase in FcγRIIIa cross-linking antibodies against the HA of H5N1 suggesting broad reactivity across group 1 and 2 influenza viruses. A high response to TIV appears to boost broadly cross-reactive FcγRIIIa cross-linking antibodies capable of recognizing avian HA proteins.

ADCC of Influenza-Infected Cells in Older Adults

To assess whether serum FcγRIIIa dimer binding and NK cell-activating antibodies induce ADCC of influenza-infected lung epithelial cells, we performed LDH release assays with pre- and postvaccination sera samples from a subset of 10 subjects randomly chosen from each group. Low and high responders by HAI demonstrated a significant rise in ADCC of B/Florida/04/2006-infected A549 cells at day 21 postvaccination, whereas the nonresponder group did not show increased ADCC post-TIV (Figure 6A). A similar trend of antibody-mediated killing by NK cells was also observed against A/Brisbane/59/2007(H1N1)-infected A549 cells; however, only high responders showed a significant increase in ADCC while nonresponder and low responder groups did not (Figure 6A). Cytotoxicity results with influenza-infected target cells correlated with both antibody-mediated FcγRIIIa dimer binding and NK cell activation (Figure 6B and C).

IgG Subclass Specificity of HA Binding Antibodies Postvaccination

IgG subclasses IgG1 and IgG3 typically bind to FcγRIIIa with higher affinities and have a greater capacity to activate NK cells and induce ADCC than IgG2 and IgG4 [32]. To determine the IgG subclasses responsible for the rise in ADCC activity post-TIV we analyzed HA-specific IgG by multiplex. The high responder group demonstrated an increase in HA-specific IgG1 and IgG3 postvaccination across all 3 TIV strains (Figure 7A and Supplementary Figure 1). High responders also demonstrated a postvaccination rise in IgG2 against the HA of A/Florida/04/2006 and an increase in IgG4 against the HAs of A/Brisbane/10/2007(H3N2) and B/Florida/04/2006 post-TIV. Nonresponders showed a rise in IgG3 to the HAs of A/Brisbane/10/2007(H3N2) and B/Florida/04/2006 (Figure 7A), but the magnitude of the increase was low relative to the high responders (Supplementary Figure 1). Thus, IgG1 and IgG3 subclasses drive TIV-induced ADCC in older adults.

Principal Component Analysis of TIV Nonresponders and High Responders

Principal component analysis comparing baseline responses between nonresponders and high responders demonstrated weak clustering (Figure 7B). The main features associated with nonresponders were male gender, advanced age, IgG2, and IgG4, while high responders were more associated with FcγRIIIa dimer binding antibodies, NK cell activating antibodies, HAI antibodies, and IgG1.

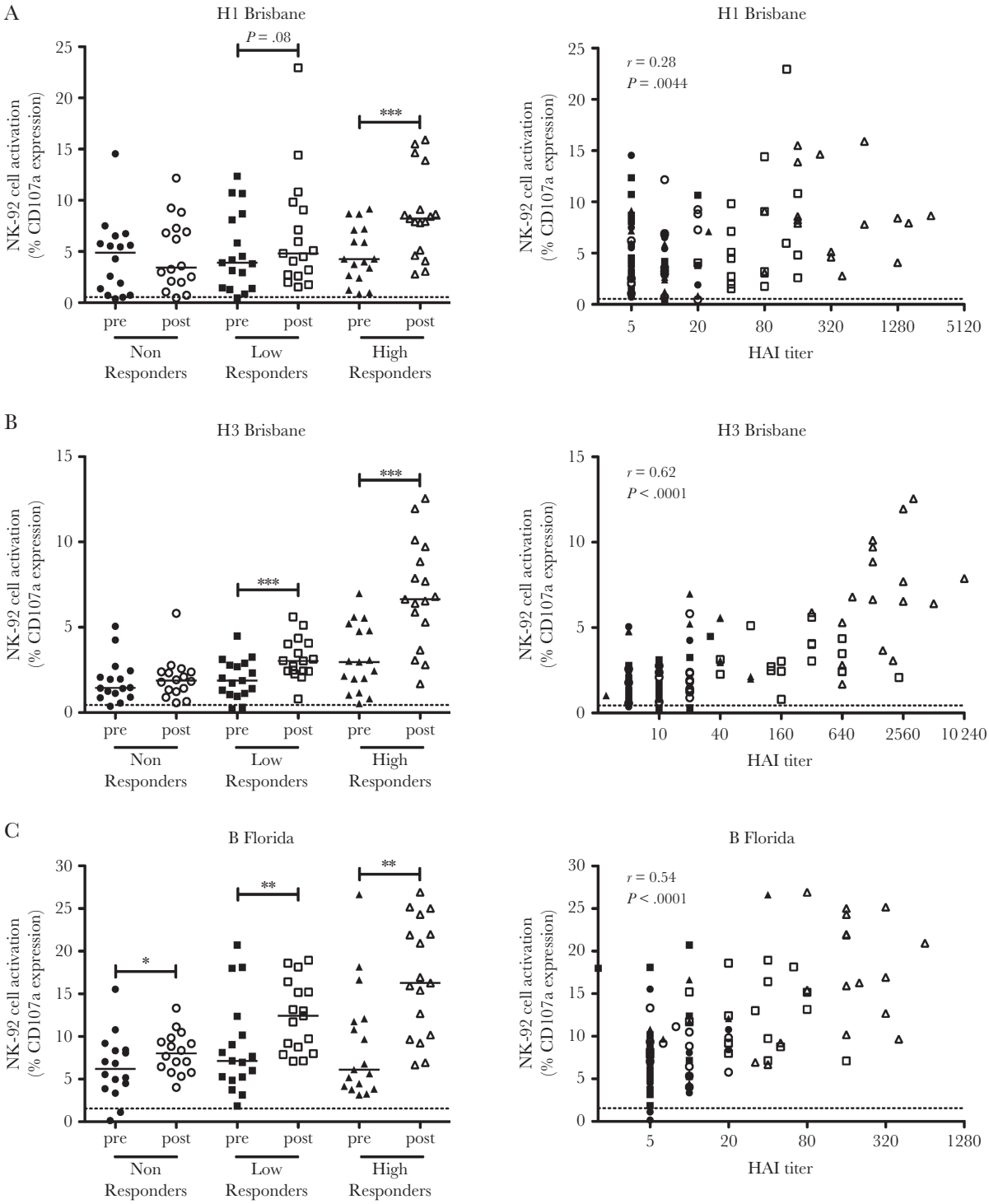

Figure 4. Postvaccination increase in natural killer (NK) cell activating antibodies against the hemagglutinins (HAs) of the 2008/2009 seasonal trivalent influenza vaccine (TIV) viruses in sera from older adult responders. NK cell activating antibodies against HA proteins from A/Brisbane/59/2007(H1N1) virus (*A*), A/Brisbane/10/2007(H3N2) virus (*B*), and B/Florida/04/2006 virus (*C*) are shown (symbols as in Figure 3). Spearman correlations between serum NK cell activating antibodies and hemagglutination inhibiting (HAI) geometric mean titers (GMTs) are also shown for the H1N1 (*A*), H3N2 (*B*), and B (*C*) TIV strains. All pre- and postvaccination sera samples were also tested for NK cell activation by antibodies against an irrelevant HIV-1 protein gp140 and background was subtracted for each serum sample. Dotted lines denote the limit of detection of the NK cell activation assay, which represents 3 times the NK cell activation in wells with HA protein but no sera. Wilcoxon-matched pairs signed rank tests were used to compare pre- and postvaccination samples. * $P < .05$, ** $P < .01$, *** $P < .001$.

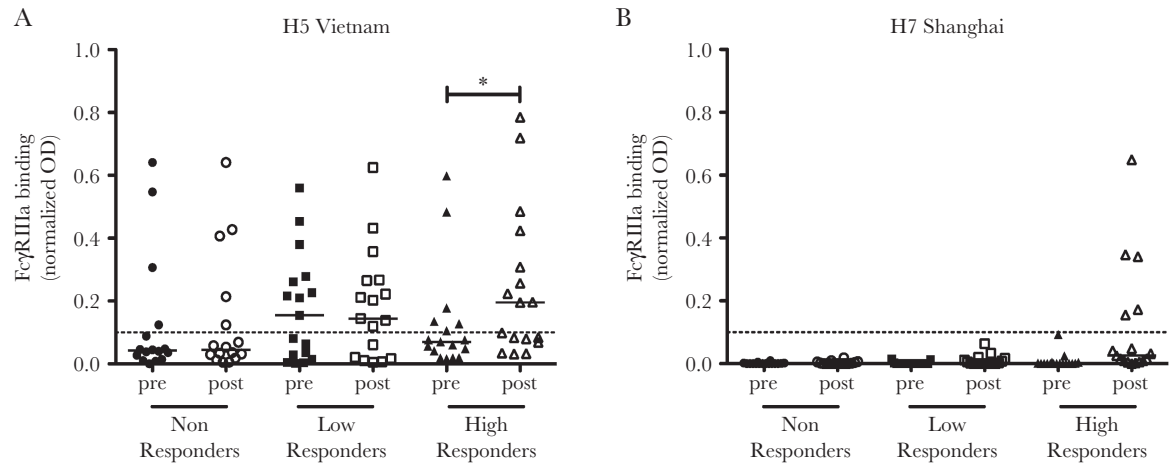

Figure 5. Increased FcγRIIIa cross-linking antibodies against hemagglutinins (HAs) from avian influenza viruses in postvaccination sera from older adult high responders. FcγRIIIa dimer binding antibodies against HA proteins from the avian A/Vietnam/1194/2004 (H5N1) virus (*A*) and the avian A/Shanghai/01/2013 (H7N9) virus (*B*) are shown. Symbols, dotted lines, controls, and statistics are as in Figure 3. * $P < .05$, ** $P < .01$, *** $P < .001$.

No clustering differences were observed when comparing baseline and postvaccination features for nonresponders (Figure 7C). However, PCA of high responders demonstrated nearly nonoverlapping profiles when comparing baseline and postvaccination measurement, with the upregulation of the majority of antibody features associated with postvaccination sera (Figure 7D).

DISCUSSION

Influenza vaccine studies typically use HAI antibodies as a correlate of vaccine-induced protection, but other markers of protection may be important [22, 23, 27–29, 33]. Recent studies in younger adults have generated contradictory results regarding the relationship between HAI and ADCC antibodies [25, 27, 29, 31, 34–37, 43]. The link between ADCC and HAI antibody responses to vaccination in older adults has not been previously studied, despite their high morbidity and mortality from seasonal influenza infections [1, 2]. We found high responding subjects aged 65 years or older by HAI also had strong ADCC responses postvaccination, as measured by FcγRIIIa dimer binding, NK cell activation, and killing of influenza-infected cells. This finding is surprising as recent work has demonstrated that both HAI$^+$ mAbs and serum HAI antibodies, elicited by an adjuvanted H5N1 vaccine, were inhibitory to ADCC [25, 31, 35]. HAI antibodies can block critical interactions between HA on the surface of influenza-infected cells and sialic acid on immune effector cells, thereby reducing ADCC activity [25, 31, 35]. Based on these data, elevated HAI titers postvaccination should result in decreased serum ADCC activity in older adult TIV responders, but increased ADCC activity was detected even in the presence of high HAI titers. Our results are in broad agreement with a number of other studies performed in influenza vaccinated children and adults [29, 34]. There are several plausible explanations for these conflicting results, including differences in the ADCC readout (influenza-infected cell killing or CD107a vs luciferase reporter), the effector cell type used (NK-92-FcγRIIIa-GFP vs Jurkat cells), the degree of target cell infection (antigen density on surface of target cells), and the vaccine administered (single dose unadjuvanted TIV vs adjuvanted prime-boost H5N1 vaccine). Whether technical differences can alter the observed relationship between ADCC and HAI antibodies warrants further investigation.

Adults have broadly reactive HA-specific ADCC antibodies capable of recognizing influenza viruses to which they have no HAI titer [27, 28]. We found that >70% of older adults showed detectable prevaccination levels of ADCC-mediating antibodies against the H1N1 and B TIV strains in the absence of elevated HAI titers (HAI < 40). Whether these "ADCC positive, HAI negative" subjects have partial protective immunity to seasonal influenza is of great interest and should be examined in larger vaccine efficacy trials with virological endpoints. Prevaccination levels of HA-specific ADCC-mediating antibodies were notably lower to the H3N2 vaccine strain, potentially due to more rapid antigenic drift of H3N2 influenza viruses compared to H1N1 and B viruses. At baseline, 39% of older adults also had detectable FcγRIIIa cross-linking antibodies against the HA of an avian H5N1 influenza virus, to which direct exposure was unlikely. Future studies could be performed with target cells stably expressing H5 and H7 to determine whether the same antibodies mediate ADCC against group 1 and 2 HAs. Preclinical work suggests cross-reactive HA antibodies may provide older adults with some level of Fc-mediated protection against influenza infection in the absence of vaccine-induced HAI [20, 21, 25, 26, 31]. Recent work showed that a small subset of human subjects (n = 3) with high titers of ADCC antibodies (≥320) prior to experimental A/Wisconsin/67/2005(H3N2) influenza

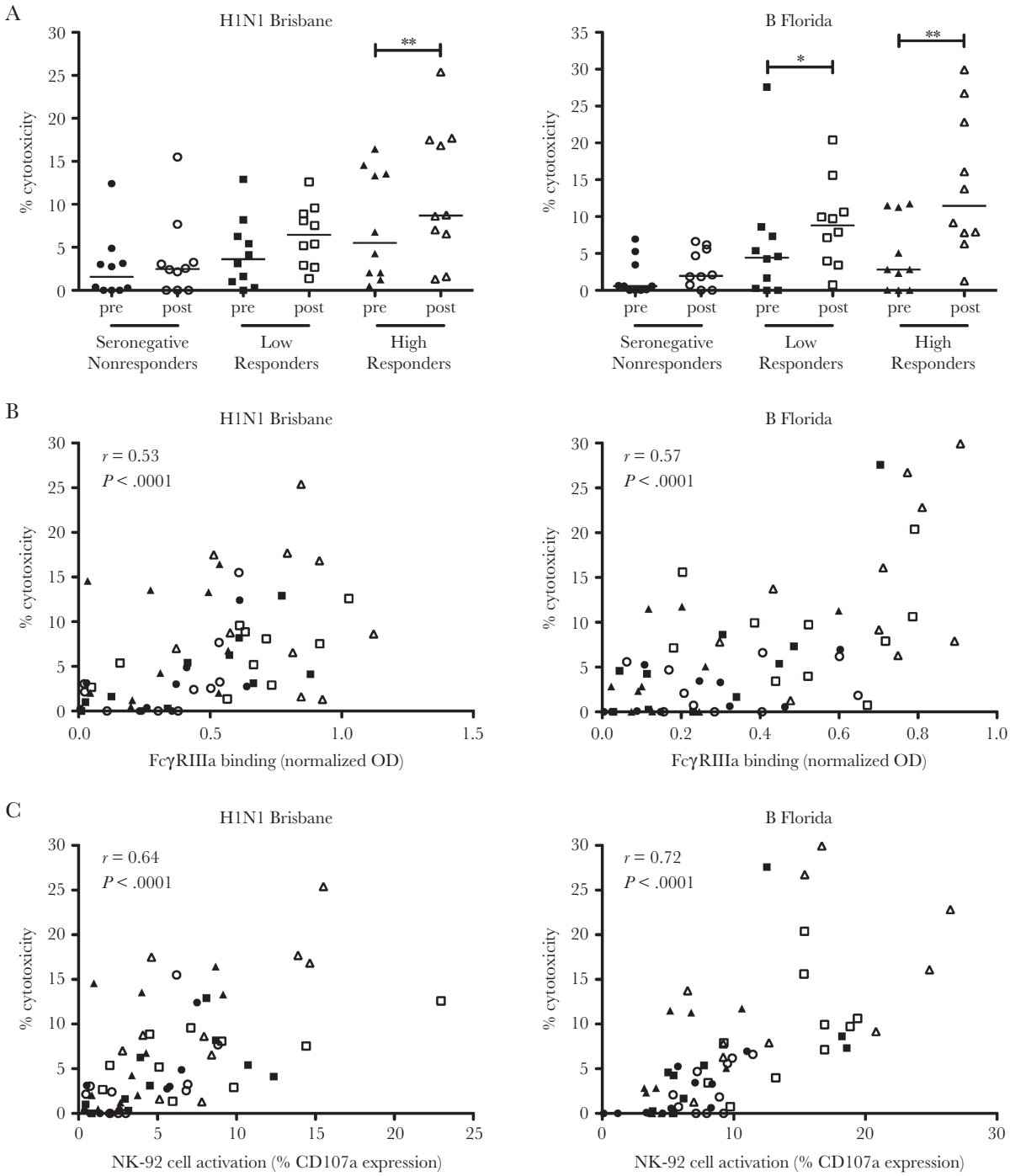

Figure 6. Greater antibody-dependent cellular cytotoxicity (ADCC) of influenza-infected lung epithelial cells with postvaccination sera from older adult high responders. *A*, ADCC of A/Brisbane/59/2007(H1N1)-infected and B/Florida/04/2006-infected A549 target cells by NK-92-FcγRIIIa-GFP effector cells is shown in the presence of pre- and postvaccination sera from older adult nonresponders, low responders and high responders (symbols as in Figure 3). ADCC was measured by the release of lactate dehydrogenase from target cells. Wilcoxon-matched pairs signed rank tests were used to compare pre- and postvaccination samples. Spearman correlations of ADCC with both FcγRIIIa dimer binding antibodies (*B*) and natural killer (NK) cell activating antibodies (*C*) are shown. * $P < .05$, ** $P < .01$.

infection demonstrated lower symptom scores and less detectable virus postinfection, suggesting that ADCC antibodies may play a role in reducing the severity of seasonal influenza [29]. Antibodies targeting other influenza antigens, including neuraminidase, matrix, and nucleoprotein, can mediate ADCC and may also be partially responsible for killing influenza-infected cells [20, 36, 44–46].

The heterogeneity of seasonal TIV response within the older population presents a formidable challenge in achieving widespread disease prevention and to date no serological predictors

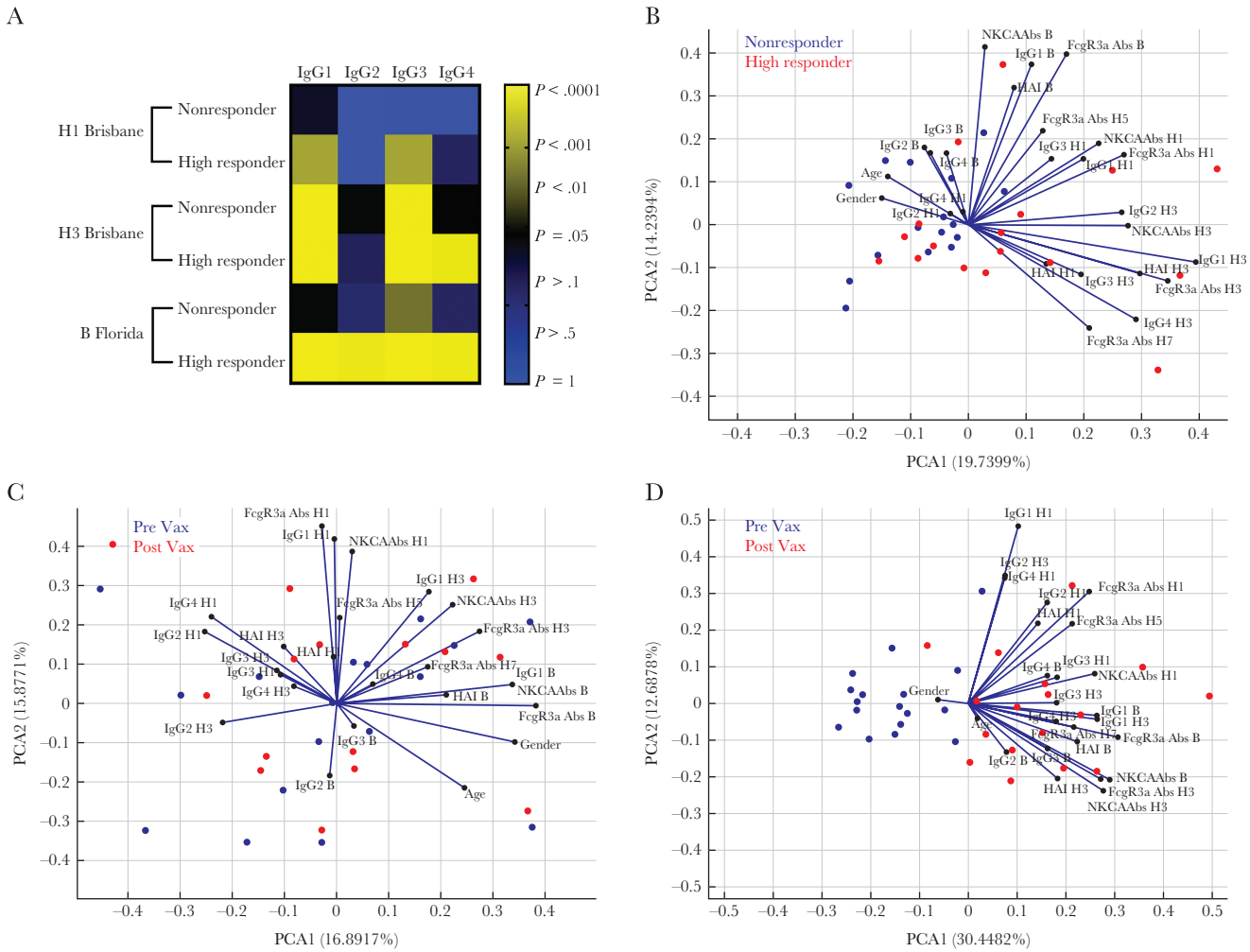

Figure 7. Heat map of IgG subclass multiplex and principal component analysis (PCA) for older adult vaccine nonresponders and high responders. *A*, Heat map of Wilcoxon-matched pairs signed rank test *P* values. Each square is a *P* value representing the statistical significance of the postvaccination increase in median fluorescence intensity (MFI) for IgG subclasses 1–4 in sera from older adult nonresponders and high responders. IgG subclasses were tested against the 3 vaccine strains of influenza: A/Brisbane/59/2007(H1N1), A/Brisbane/10/2007(H3N2), and B/Florida/04/2006. Yellow squares represent *P* values < .05 and indicate a statistically significant increase in the IgG subclass day 21 post-TIV. Blue squares represent *P* values > .05 and indicate no significant increase in an IgG subclass post-TIV. *B*, PCA using 25 features measured at baseline demonstrated weak clustering differences between nonresponders (blue dots) and high responders (red dots). PCA of nonresponders (*C*) and high responders (*D*) were conducted including the same 25 features measured at baseline (Pre Vax; blue dots) and postvaccination (Post Vax; red dots). Location of labeled features (loadings plot with black dots) overlaid upon subjects reflect their distribution. Variance for principal component 1 (PCA1) and principal component 2 (PCA2) are described on x and y axes, respectively.

of vaccine responsiveness have been identified. In each ADCC assay performed, older adults demonstrated a baseline breadth of serum ADCC activity, but as previously reported [29] this did not predict HAI response to vaccination (Supplementary Figure 2). Yet, PCA using 25 features measured in prevaccination sera samples revealed that the main features associated with high responders by HAI were dimeric FcγRIIIa binding antibodies, NK cell activating antibodies, and IgG1 (Figure 7B). In contrast, nonresponders were more associated with advanced age, male gender, IgG2, and IgG4 (Figure 7B). However, due to weak clustering and low variance (34.1%) we cannot assert that FcγRIIIa cross-linking, NK cell activation, breadth to avian HAs, or IgG subclass composition are strongly predictive of vaccine responsiveness. Interestingly, the PCA results suggest that older men generally exhibit poor TIV responsiveness, consistent with previous findings on gender differences [47, 48]. Comprehensive screening of sera for antibodies and other relevant soluble factors may provide further insight into vaccine-induced immunity in older adults.

In summary, poor HAI responses to TIV in older adults likely contribute to increased morbidity and mortality in this age group. A sizeable portion of older adults have ADCC antibodies against seasonal vaccine strains of influenza at baseline, which could provide a degree of protective immunity in the absence of HAI. Overall, the ADCC response to vaccination tracked closely with HAI in older adults, which was unexpected as recent studies

have suggested that HAI antibodies are inhibitory to influenza-specific ADCC. There is a need for both improved markers of immunological responsiveness to the current influenza vaccine as well as more effective influenza vaccines for older adults.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. Sarina Carmuglia and Steven Rockman are employees of Seqirus, a CSL company, that manufactures influenza vaccines. The other authors have no conflicts of interest to declare. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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