

Antibody Responses with Fc-Mediated Functions after Vaccination of HIV-Infected Subjects with Trivalent Influenza Vaccine

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ABSTRACT

This study seeks to assess the ability of seasonal trivalent inactivated influenza vaccine (TIV) to induce nonneutralizing antibodies (Abs) with Fc-mediated functions in HIV-uninfected and HIV-infected subjects. Functional influenza-specific Ab responses were studied in 30 HIV-negative and 27 HIV-positive subjects immunized against seasonal influenza. All 57 subjects received the 2015 TIV. Fc-mediated antihemagglutinin (anti-HA) Ab activity was measured in plasma before and 4 weeks after vaccination using Fc-receptor-binding assays, NK cell activation assays, and phagocytosis assays. At baseline, the HIV-positive group had detectable but reduced functional Ab responses to both vaccine and nonvaccine influenza antigens. TIV enhanced Fc-mediated Ab responses in both HIV-positive and HIV-negative groups. A larger rise was generally observed in the HIV-positive group, such that there was no difference in functional Ab responses between the two groups after vaccination. The 2015 TIV enhanced functional influenza-specific Ab responses in both HIV-negative and HIV-positive subjects to a range of influenza HA proteins. The increase in functional Ab responses in the HIV-positive group supports recommendations to immunize this at-risk group.

IMPORTANCE

Infection with HIV is associated with increasing disease severity following influenza infections, and annual influenza vaccinations are recommended for this target group. However, HIV-infected individuals respond relatively poorly to vaccination compared to healthy individuals, particularly if immunodeficient. There is therefore a need to increase our understanding of immunity to influenza in the context of underlying HIV infection. While antibodies can mediate direct virus neutralization, interactions with cellular Fc receptors may be important for anti-influenza immunity *in vivo* by facilitating antibody-dependent cellular cytotoxicity (ADCC) and/or antibody-dependent phagocytosis (ADP). The ability of seasonal influenza vaccines to induce antibody responses with potent Fc-mediated antiviral activity is currently unclear. Probing the ADCC and ADP responses to influenza vaccination has provided important new information in the quest to improve immunity to influenza.

Seasonal influenza infections cause significant illness and deaths worldwide, with immunocompromised people such as those infected with human immunodeficiency virus (HIV) at high risk for developing influenza-related complications (1, 2). The main strategy to prevent seasonal influenza is through a trivalent inactivated influenza vaccine (TIV), and there is evidence that TIV shows some efficacy in HIV⁺ subjects (3). The TIV contains two influenza type A strains (H1N1, H3N2) and one influenza type B strain and is thought to act by primarily inducing neutralizing antibodies (NAbs) to the viral hemagglutinin (HA) (4). These NAbs are generally highly specific to the influenza virus strains in the vaccine, but because influenza viruses constitutively mutate, vaccines to seasonal influenza are updated yearly to maintain their effectiveness (5).

Antibodies (Abs) that induce Fc-dependent cellular functions are generated following influenza infections (6, 7). Fc-dependent functions include antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADP). ADCC and ADP are initiated when Abs recognize and bind to antigens on the surface of an infected cell and engage effector cells bearing receptors for the Fc portion of IgG (FcγR). The effector cells,

including natural killer (NK) cells and monocytes, may then be activated to kill the infected target cell (5, 8). In addition, free virions bound by Abs are a key target for ADP. ADCC is mediated primarily through FcγRIIIa (CD16a), which is present in higher-affinity (V158) and lower-affinity (F158) variants (9), whereas ADP is mediated primarily through FcγRIIa (CD32a) (10).

Studies in both mice and macaques suggest that ADCC may play an important role in the control of influenza (11, 12). However, the extent to which TIV induces ADCC and other Fc-medi-

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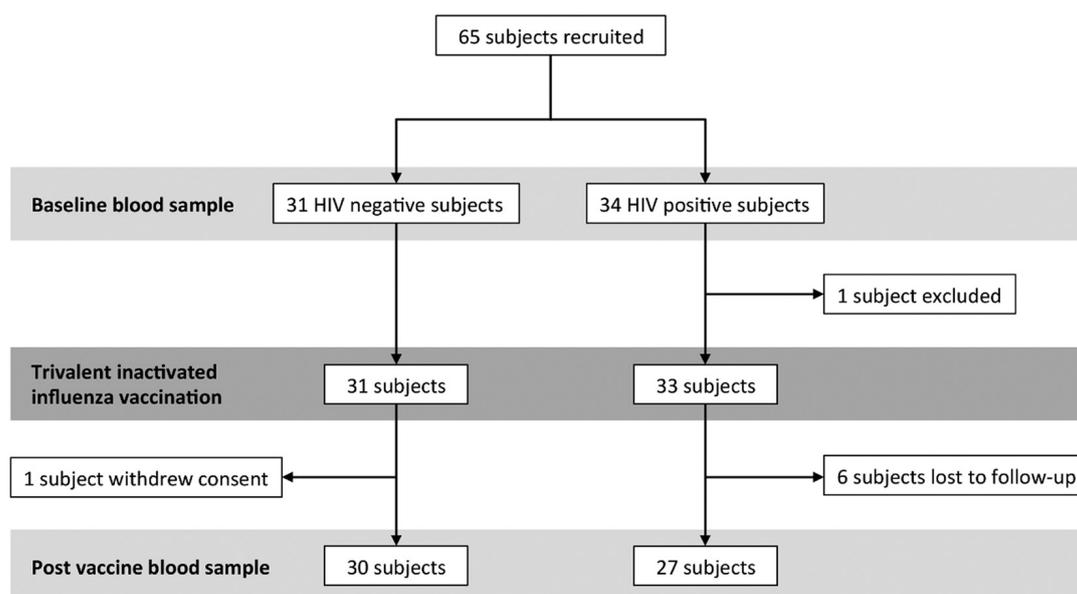


FIG 1 Study enrollment flowchart.

ated Ab functions such as ADP has not been well studied. A seasonal TIV vaccine failed to elicit ADCC Abs in influenza-naive pigtail macaques (13); however, studies in animal models do not recapitulate the complex immune landscape from multiple prior influenza exposures in human adults. Since HIV can cause widespread B cell dysfunction (14), there is a need to understand non-NAb responses to influenza in subjects with HIV infection and how these are modulated by vaccination.

Here we recruited two cohorts of HIV-negative and HIV-positive subjects and studied functional influenza-specific Ab responses employing a number of novel assays. Two new influenza virus strains were introduced into the 2015 TIV [A/Switzerland/9715293/2013 (H3N2)-like virus and B/Phuket/3073/2013-like virus] in the Southern Hemisphere, and this vaccine is now being used in the Northern Hemisphere (<http://www.who.int/influenza/vaccines/virus/recommendations/en/>). The introduction of new strains in the 2015 TIV allowed us to study functional Abs to antigenically variant influenza virus strains in human adults.

MATERIALS AND METHODS

Influenza vaccine cohort. We concurrently recruited 30 healthy and 27 HIV-infected adults from the Doherty Institute for Infection and Immunity at the University of Melbourne, Parkville, Australia, and from the Melbourne Sexual Health Centre (MSHC) in Melbourne, Australia (Fig. 1 and Table 1). Based on data regarding the variability of influenza ADCC in HIV-uninfected subjects prior to vaccination, we predicted that 30 subjects per group would provide adequate power (80%, 2- α = 0.05) to detect 2-fold differences in ADCC responses between baseline responses in HIV-negative and HIV-positive subjects. Subjects received an intramuscular injection of TIV (Fluvax; bio-CSL, Parkville, Australia) containing 15 μ g of hemagglutinin (HA) from A/California/7/2009 (H1N1), A/South Australia/55/2014 (H3N2), and B/Phuket/3073/2013. A/South Australia/55/2014 is an A/Switzerland/9715293/2013 (H3N2)-like virus selected for inclusion in trivalent influenza vaccines for the Southern Hemisphere, with only a single K207R mutation (H3 numbering) differentiating these two antigenically similar strains. Blood samples were collected at baseline and a mean of 28 days (interquartile range, 27 to 29; range, 19 to 45) after vaccination. The study protocol and procedures were

approved by the Alfred Health and University of Melbourne Human Ethics Committees (IDs 432/14 and 1443420). All subjects provided written informed consent.

Recombinant proteins. Simian immunodeficiency virus (SIV) gp120 and purified HA from A/Switzerland/9715293/2013 (H3N2), A/Perth/16/2009 (H3N2), A/California/7/2009 (H1N1), A/New Caledonia/20/1999 (H1N1), B/Phuket/3073/2013 (Yamagata), and B/Florida/4/2006 (Yamagata) were purchased from Sinobiological, Beijing, China. SIV gp120 protein was included in each experiment as a negative control.

HI assays. Hemagglutination inhibition (HI) assays were performed as previously described (15) using A/South Australia/55/2014 (H3N2), B/Phuket/3073/2013, A/California/7/2009 (H1N1), and A/New Caledonia/20/1999 (H1N1) viruses. Briefly, HI activity in serum samples was assessed using 1% turkey erythrocytes (H1N1 subtypes and type B influenza) or 1% guinea pig erythrocytes (H3N2 subtypes). Serum samples were titrated from a starting dilution of 1:10 to 1:1,280 in a phosphate-buffered saline (PBS) solution. HI titers were expressed as the reciprocal of the highest dilution of serum at which hemagglutination was completely inhibited.

Fc γ R dimer-binding assay. When multiple IgG Abs bind simultaneously to antigen and present their Fc regions in close proximity to each other, they cluster cellular Fc γ Rs, resulting in cell activation and effector function. A correlate of this “effector-activating” capacity of IgG Abs can be measured *in vitro* by novel assays that use dimers of the ligand binding domains of Fc γ RIIa or Fc γ RIIIa (see Fig. 3A) (B. D. Wines, H. A. Vander-ven, S. E. Esparon, A. B. Kristensen, S. J. Kent, and P. M. Hogarth, unpublished data). Maxisorp enzyme-linked immunosorbent assay (ELISA) plates (96-well plates; Nunc, Rochester, NY) were coated with 50 ng/well of influenza HA or SIV gp120 (Sinobiological) in PBS overnight at 4°C. Wells were washed with PBS containing 0.05% Tween 20 (U-CyTech) and blocked with PBS containing 1 mM EDTA and 1% human serum albumin for 1 h at 37°C. Plates were washed and incubated with 1:20 diluted plasma (1:40 for A/New Caledonia/20/1999-coated wells) for 1 h at 37°C and then with 0.2 μ g/ml of biotinylated Fc γ RIIa dimer or 0.1 μ g/ml of biotinylated Fc γ RIIIa dimer diluted in PBS with 1 mM EDTA and 1% bovine serum albumin (PBS-EDTA-BSA; Sigma-Aldrich) for 1 h at 37°C. Subsequently, 100 ng/ml of Pierce high-sensitivity streptavidin-horseradish peroxidase (HRP; Thermo Scientific, Pittsburgh, PA) diluted in PBS-EDTA-BSA was added for 1 h at 37°C. Lastly, TMB (3,3',5,5'-tetramethylbenzidine) substrate (Sigma-Aldrich) was added, and color development was stopped

TABLE 1 Baseline characteristics of participants

Characteristic	HIV negative (<i>n</i> = 30)	HIV positive (<i>n</i> = 27)
Age (yr)		
Mean	40.4	41.4
Range	22–55	26–54
No. (%) of male gender	13 (43.3)	26 (96.3)
Duration of HIV infection (yr)		
Mean		8.1
Range		1.0–20.8
No. (%) on antiretroviral therapy		26 (96.3)
CD4 ⁺ T cell count (per μ l)		
Mean		729
Range		257–1843
Nadir CD4 ⁺ T cell count (per μ l)		
Mean		345
Range		73–852
Viral load (HIV RNA copies/ml plasma)		<20 ^a
Days between Fluvax vaccine and 2nd blood sampling		
Mean	27	29
Interquartile range	22–29	27.5–31.5
No. (%) who received influenza vaccinations in the last 5 years ^b		
Unknown	2 (6.7)	3 (11.1)
0 vaccinations	2 (6.7)	2 (7.4)
1 vaccination	6 (20.0)	2 (7.4)
2 vaccinations	4 (13.3)	5 (18.5)
≥ 3 vaccinations	16 (53.3)	15 (53.3)

^a Three subjects had detectable viral loads (25, 55, and 7,010 RNA copies/ml plasma).

^b Self-reported data and from medical records from MSHC if available.

with 1 M hydrochloric acid. Absorbance was read at 450 nm. Data were normalized using the signal from Fc γ R dimer binding to control wells containing 5 μ g/ml human intravenous immunoglobulin (IVIg) (Sandozglobulin; CSL Behring, Australia) diluted in PBS-EDTA-BSA and directly absorbed to the plate.

Plate-bound natural killer (NK) cell activation assay. NK cell activation was measured by the ability of HA-specific Abs to induce NK-92 cell expression of CD107a (see Fig. 5A). The human NK-92 cell line (16) constitutively expressing the high-affinity variant of Fc γ RIIIa (V158) was cultured as previously described (17). Maxisorp ELISA plates (96-well; Nunc, Rochester, NY) were coated with 600 ng/well of influenza HA or SIV gp120 proteins in PBS overnight at 4°C. Wells were washed and blocked in PBS containing 5% bovine serum albumin and incubated with heat-inactivated 1:40 diluted plasma for 2 h at 37°C. NK-92 cells (2×10^5) were added to each well and incubated at 37°C for 5 h. Anti-human CD107a-allophycocyanin (clone H4A3; BD Biosciences) and 1 mM EDTA were added to the cells for 30 min at room temperature in the dark. NK-92 cells were washed twice with PBS, fixed with 1% formaldehyde, and acquired on the LSR Fortessa flow cytometer (BD Biosciences). Analysis was performed using FlowJo X 10.0.7r2 software (FlowJo LLC, Ashland, OR). Average background (SIV gp120-coated wells) was 1.5% NK-92 cells expressing CD107a.

ADP assay. The antibody-dependent phagocytosis assay (10) was adapted for influenza virus (see Fig. 6A). Briefly, fluorescein isothiocyanate (FITC)-labeled NeutrAvidin FluoSpheres (1 μ m; Invitrogen) were coated with a Cy5-fluorescent internalization probe (FIP) (5'-Cy5-TCA

GTTCAGGACCCTCGGCT-3Bio-3'; Integrated DNA Technologies). After 10 min, excess FIP was washed. FIP-coated beads were incubated overnight with either 0.25 μ g/ μ l biotinylated HA from A/Switzerland/9715293/2013 (H3N2) or biotinylated SIV gp120 (Sinobiological). Beads were washed to remove unbound protein and diluted 1:100 in PBS containing 2% BSA. Ten microliters coated beads was mixed with 10 μ g/ml purified IgG from plasma for 2 h at 37°C. IgG was purified using the protein G HP Multitrap kits (GE Healthcare, Buckinghamshire, United Kingdom) as previously described (10). THP-1 cells (1×10^5), a monocytic cell line, were added to opsonized beads and cultured 16 h at 37°C. Cy5 fluorescence from surface-bound beads was blocked by adding the complementary quenching probe (5'-AGCCGAGGGTCCTGAACTGABHQ2-3'). Cells were fixed and analyzed by flow cytometry as described above. Average background (SIV gp120-coated beads) was 7.5% cells with internalized beads.

Statistical analysis. Statistical analysis was performed with Prism GraphPad version 6.0g (GraphPad Software, San Diego, CA). Data were analyzed by the Mann-Whitney U test, the Wilcoxon matched-pair signed-rank test, or the Spearman correlation coefficient as indicated in the figure legends. Data are presented as means \pm standard errors of the means (SEM).

RESULTS

Influenza vaccination cohorts. People infected with HIV are at greater risk for severe influenza infection and respond more

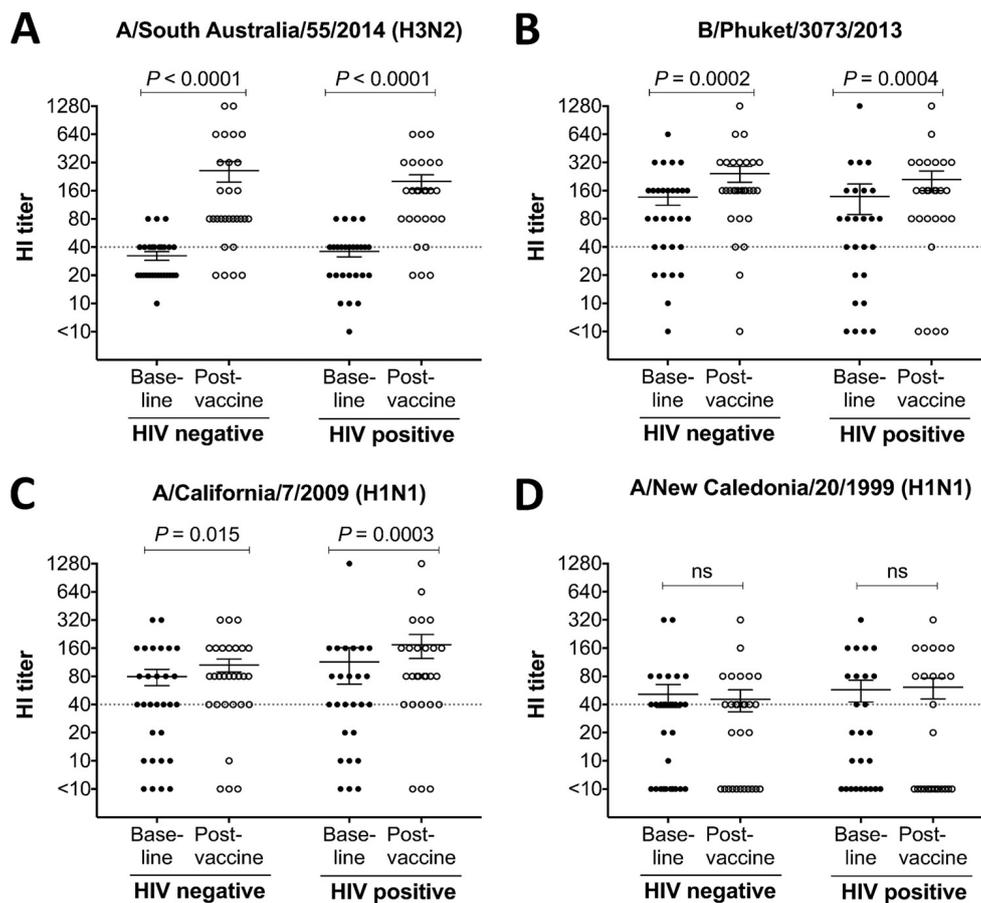


FIG 2 Hemagglutination inhibition (HI) assay. Serum HI titers against vaccine influenza virus strains A/South Australia/55/2014 (H3N2) (A), B/Phuket/3073/2013 (B), and A/California/7/2009 (H1N1) (C) and a nonvaccine strain, A/New Caledonia/20/1999 (H1N1) (D). Baseline and postvaccine data were compared using the Wilcoxon matched-pair test. Lines and error bars indicate means and SEM, respectively. A cutoff HI titer of ≥ 40 (dashed line) was used to assess positive responses.

poorly to influenza vaccination, particularly if immunodeficient (18, 19). To investigate if HIV infection alters ADCC and ADP responses to vaccination, we concurrently recruited cohorts of HIV-infected ($n = 27$) and HIV-uninfected ($n = 30$) subjects to receive the 2015 Southern Hemisphere TIV (Fig. 1). The cohorts were well matched for age and prior influenza vaccinations (Table 1). The HIV-positive subjects were more commonly male, and all but one was on effective antiretroviral therapy (ART) with a mean CD4 T cell count of $729/\mu\text{l}$.

HI responses to 2015 TIV in HIV-positive and HIV-negative subjects. The 2015 Southern Hemisphere TIV saw the inclusion of two new circulating influenza virus strains, A/South Australia/55/2014 [an A/Switzerland/9715293/2013 (H3N2)-like virus] and a B/Phuket/3073/2013 virus. The 2015 TIV retained the A/California/7/2009, the seasonal H1N1 component since 2010 (<http://www.who.int/influenza/vaccines/virus/recommendations/en/>). Prior to vaccination, 43% (13/30) of HIV-negative subjects and 58% (15/26) of HIV-positive subjects were HI seropositive (HI titer, ≥ 40) to A/South Australia/55/2014 (Fig. 2A), 80% (24/30) and 69% (18/26) to B/Phuket/3073/2013 (Fig. 2B), and 67% (20/30) and 69% (18/26) to A/California/7/2009 (Fig. 2C). After vaccination, we detected a significant increase in HI titers against all three influenza virus strains in the TIV vaccine in both the HIV-positive and HIV-negative groups (Fig. 2A to C, paired analyses).

No change in HI responses were observed against a heterologous nonvaccine H1N1 strain, A/New Caledonia/20/1999 (Fig. 2D), confirming that TIV induces primarily strain-specific NAbs. There was no significant difference between HIV-negative and HIV-positive cohorts in HI titers to any of the four influenza virus strains tested either at baseline or after vaccination. This finding contrasts with previous studies on HI responses in HIV-infected subjects (18, 20–23) and likely reflects the generally immunocompetent nature and high CD4 T cell counts of our HIV-positive cohort (24, 25).

Induction of HA-specific Fc γ RIIIa-binding Abs by TIV. There is an increasing interest in functional Ab responses to influenza, such as ADCC and ADP, although the extent to which TIV induces ADCC or ADP in humans has not been well studied. Measuring Fc-mediated Ab function can be difficult to standardize because of genotypic differences and variable activation statuses of effector cells. Further, many flow cytometry-based assays are low throughput and are not well suited for studying multiple antigens in larger cohorts. To allow ready quantification of influenza-specific Abs with Fc-mediated functions, we recently developed a high-throughput ELISA-based assay that measures the ability of HA-specific Abs to bind to a dimer of Fc γ R ectodomains (Wines et al., unpublished) (Fig. 3A). This Fc γ R dimer-binding ELISA correlates strongly with measures of Fc-mediated NK cell

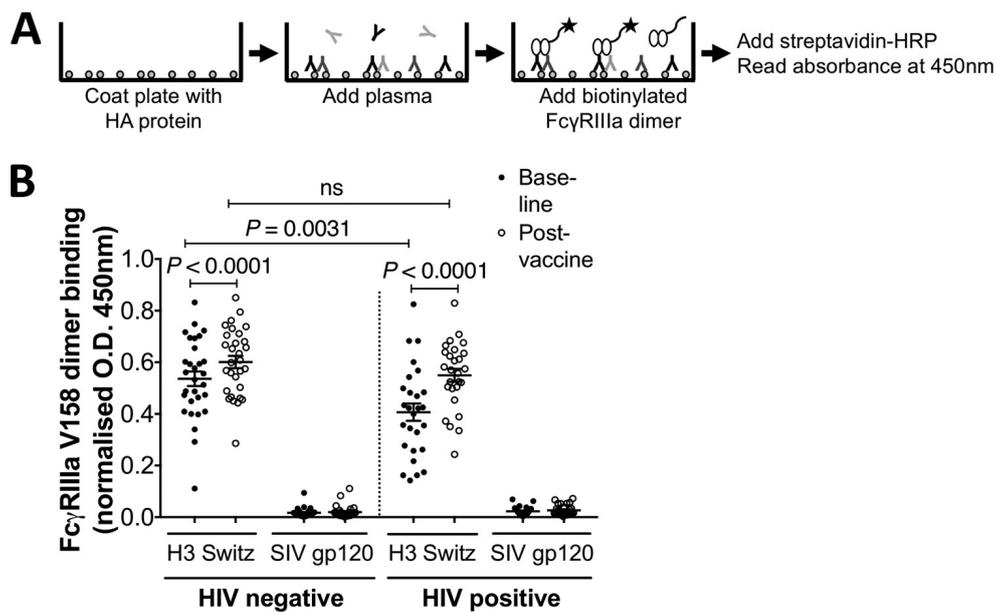


FIG 3 Fc γ RIIIa dimer-binding assay. (A) Assay setup. Influenza HA protein (50 ng/well) was coated onto a 96-well plate before adding diluted plasma (containing IgGs). Subsequently, biotinylated Fc γ RIIIa dimers were added, and binding between Abs and Fc γ RIIIa dimers was measured using HRP-conjugated streptavidin, TMB, and HCl. Absorbance was measured at 450 nm. (B) Fc γ RIIIa-V158-binding Ab responses to HA from A/Switzerland/9715293/2013 (H3N2) (H3 Switz) and SIV gp120 protein. Responses are normalized to 5 μ g/ml of IVIG. Plasma was diluted 20 times before analysis. Baseline and postvaccine data were compared using the Wilcoxon matched-pair test. HIV-negative and HIV-positive groups were compared using the Mann-Whitney test. *P* values of <0.05 were considered significant. Lines and error bars indicate means and SEM, respectively.

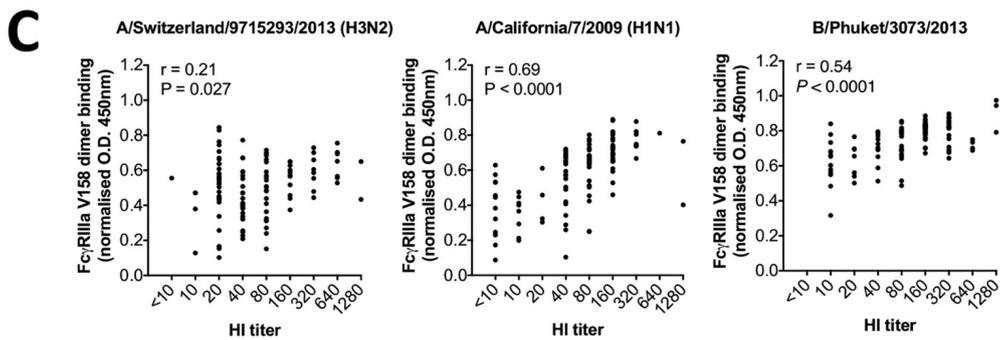
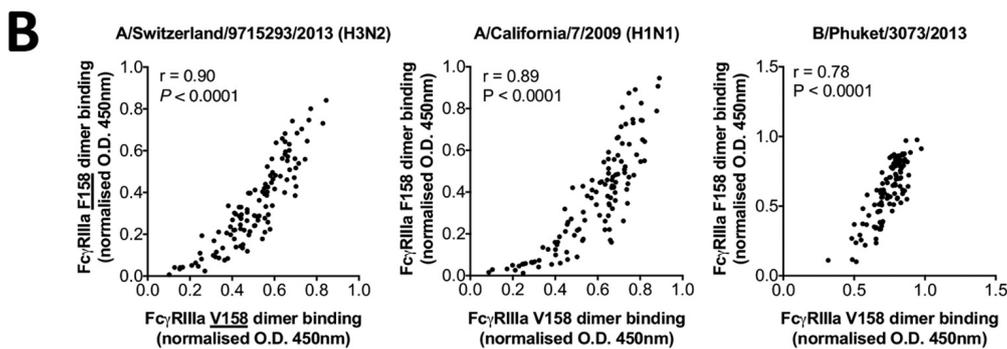
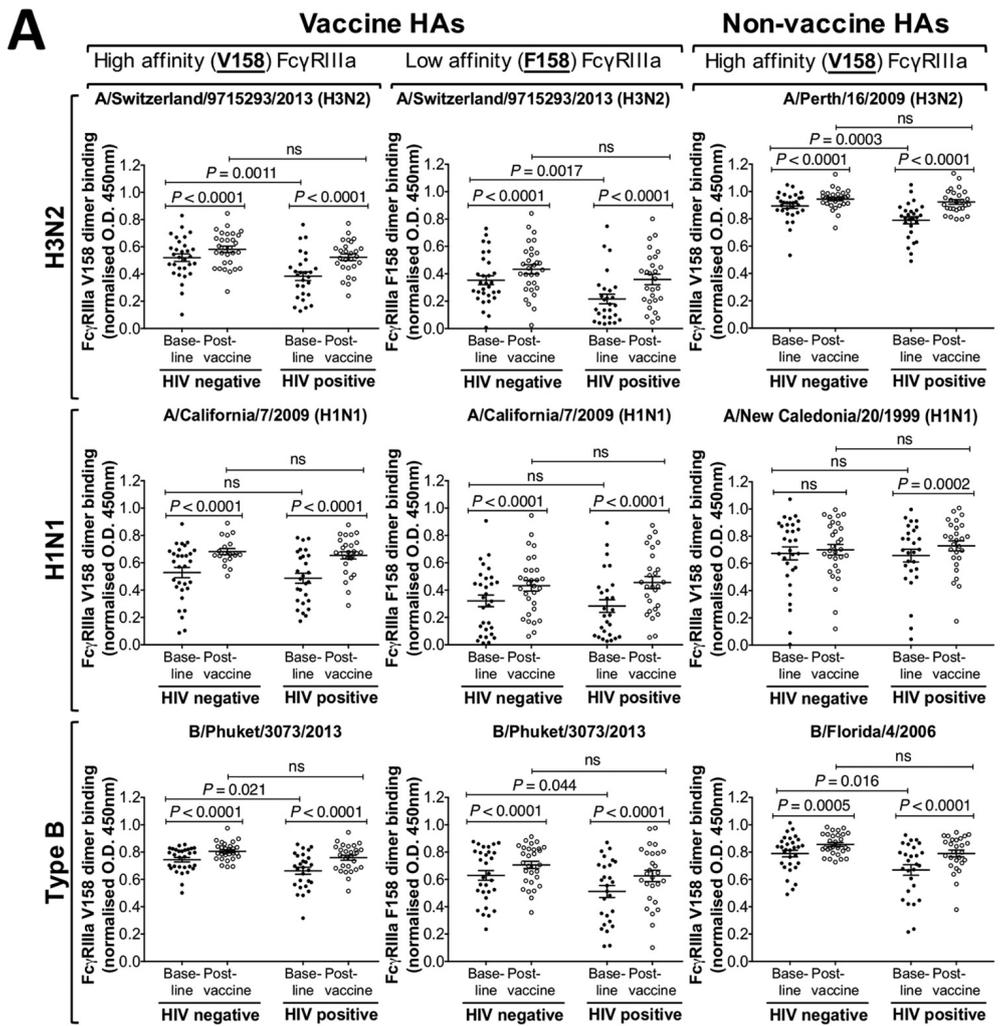
function (Wines et al., unpublished). We first assessed the capacity of HA-specific Abs to bind to the high-affinity Fc γ RIIIa-V158 dimer in vaccine recipients (Fig. 3B). Background Fc dimer-binding responses to the SIV gp120 control were low (mean, 0.02; range, 0.001 to 0.1) in both cohorts and at both time points compared to the responses to A/Switzerland/9715293/2013 HA. Background was similarly and consistently low for all other antigens tested (mean, <0.01), and binding results are subsequently presented with the background subtracted. Interestingly, baseline responses to the two new vaccine strains, A/Switzerland/9715293/2013 (antigenically matched to A/South Australia/55/2014) and B/Phuket/3073/2013, were significantly lower in the HIV-positive group than in the HIV-negative group ($P = 0.0011$ and $P = 0.021$, respectively) (Fig. 4A, left panel). After vaccination, we observed a significant rise in Fc γ RIIIa-V158-binding Abs to all three vaccine strains for both the HIV-negative and HIV-positive cohorts ($P < 0.0001$ for all). No significant difference in Fc γ RIIIa-V158-binding Abs between the HIV-negative and HIV-positive subjects was detected after vaccination, reflecting a larger increase in Fc γ RIIIa-V158-binding Abs in the HIV-positive group. Thus, these data suggest that the 2015 TIV induces Ab responses to vaccine-influenza antigens that are capable of engaging Fc γ RIIIa-V158.

The use of recombinant dimeric Fc γ Rs allows the interaction of different receptor forms to be studied. Indeed, more people are homozygous for the lower-affinity form (F158) of Fc γ RIIIa than for the higher-affinity form (V158) (9). We therefore measured the binding of HA-specific Abs to the Fc γ RIIIa-F158 dimer (Fig. 4A, center panel). Consistent with our Fc γ RIIIa-V158-binding data, we observed reduced Fc γ RIIIa-F158-binding Abs in HIV-positive subjects at baseline to A/Switzerland/9715293/2013 and B/Phuket/3073/2013 ($P = 0.0017$ and $P = 0.044$, respectively). However, no significant difference in Fc γ RIIIa-F158-binding Abs

was detected after vaccination between the two groups. The 2015 TIV induced Fc γ RIIIa-F158-binding Abs in both groups and to all 3 vaccine antigens ($P < 0.0001$ for all, paired analyses pre- and postvaccination). We observed a strong positive correlation between induction of Fc γ RIIIa-V158-binding Abs and Fc γ RIIIa-F158-binding Abs to A/Switzerland/9715293/2013 ($r = 0.90$, $P < 0.0001$; Fig. 4B), A/California/7/2009 ($r = 0.89$, $P < 0.0001$; Fig. 4B), and B/Phuket/3073/2013 ($r = 0.78$, $P < 0.0001$; Fig. 4B).

The ability of non-NAbs with Fc-mediated functions to recognize HA of multiple strains is an important potential advantage of these Abs over strain-specific NAbs (26, 27). To evaluate the induction of cross-reactive Fc γ RIIIa-binding Abs by TIV, we tested the capacity of nonvaccine HA-specific Abs to bind to the Fc γ RIIIa-V158 dimer before and after vaccination (Fig. 4A, right panel). TIV vaccination significantly increased Fc γ RIIIa-V158-binding Abs to HAs from A/Perth/16/2009, an H3N2 subtype (HIV-negative and HIV positive, $P < 0.0001$), A/New Caledonia/20/1999, an H1N1 subtype (HIV positive, $P = 0.0002$), and B/Florida/4/2006 (HIV negative, $P = 0.0005$, and HIV positive, $P < 0.0001$). No significant change in Fc γ RIIIa-V158-binding Abs was seen in the HIV-negative group to HA from A/New Caledonia/20/1999. Thus, the 2015 TIV elicited broad serological responses with Fc γ RIIIa-binding activity, presumably reflecting the ability of Fc γ RIIIa-binding antibodies to bind to conserved regions of HA (26). We also observed a significant positive correlation between HI activity and Fc γ RIIIa-V158-binding activity for all three TIV vaccine strains (Fig. 4C), although the correlation was weakest for the novel H3N2 HA in the vaccine.

Induction of HA-specific NK cell line activating Abs by TIV. We next evaluated if TIV induced Abs able to elicit degranulation (as measured by CD107a expression) of NK-92 cells, a human NK cell-like cell line transduced to express the high-affinity (V158)



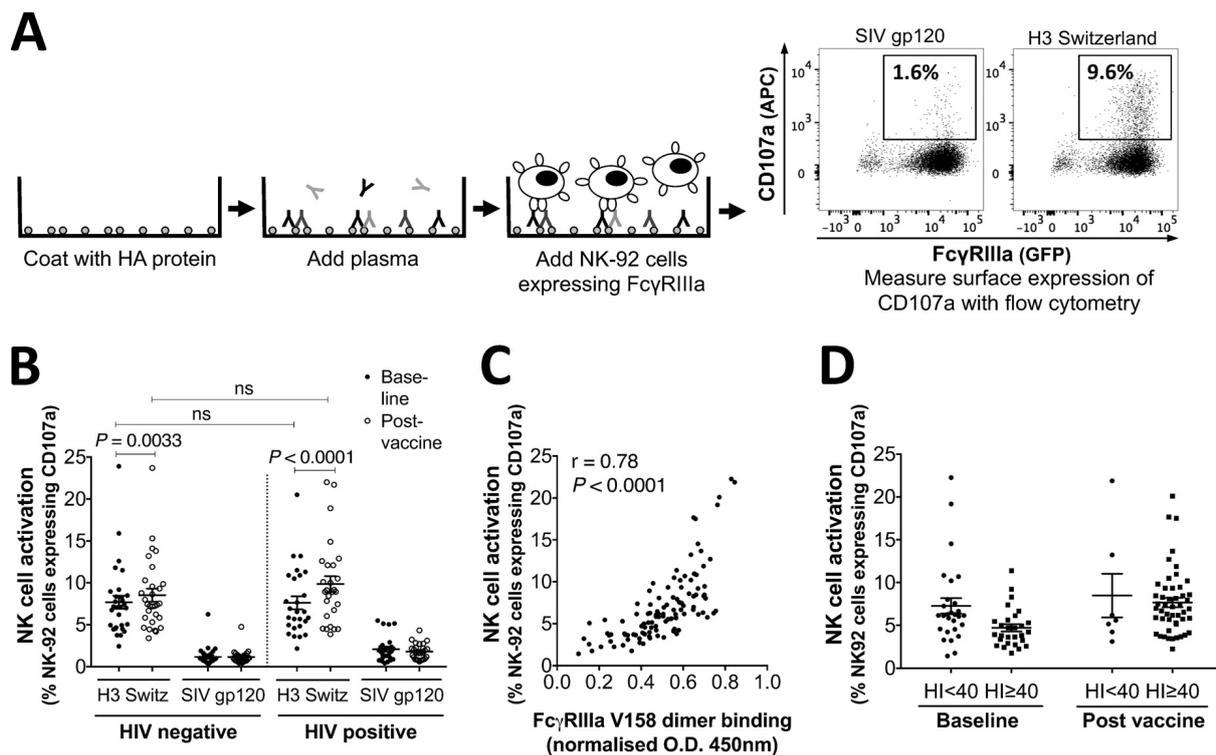


FIG 5 NK cell activation assay with the NK-92 cell line. (A) Assay setup. A 96-well plate was coated with influenza HA protein (600 ng/well) from A/Switzerland/9715293/2013 (H3N2) before diluted plasma (containing IgGs) was added. The plate was then incubated with FcγRIIIa-expressing NK-92 cells (a human NK cell line) for 5 h at 37°C, after which NK-92 cell activation was analyzed with flow cytometry by measuring the percentage of CD107a-expressing NK-92 cells. SIV gp120 protein (600 ng/well) was included in each experiment as a negative control. (B) The NK-92 cell-activating Ab response to A/Switzerland/9715293/2013, a new antigen in the 2015 TIV, and to SIV gp120 protein. NK cell activation is measured as the percentage of CD107a-expressing NK-92 cells. Plasma was diluted 40 times before analysis. Baseline and postvaccine data were compared using the Wilcoxon matched-pair test. HIV-negative and HIV-positive groups were compared using the Mann-Whitney test. *P* values of <0.05 were considered significant. Lines and error bars indicate means and SEM, respectively. (C) Correlation between FcγRIIIa-V158-binding Abs and NK-92 cell-activating Abs to HA from A/Switzerland/9715293/2013, analyzed with nonparametric Spearman correlation. Background-subtracted data are presented in the graph. Results for all HIV-negative and HIV-positive subjects (baseline and postvaccine) are shown in the graph. (D) NK-92 cell-activating Ab response to HA from A/Switzerland/9715293/2013 in the absence (HI < 40) and presence (HI ≥ 40) of HI Abs to A/South Australia/55/2014, an A/Switzerland/9715293/2013-like virus. Background-subtracted data are presented in the graph.

isoform of FcγRIIIa (Fig. 5A). We studied responses to HA from A/Switzerland/9715293/2013 (H3N2), since this antigen in the 2015 TIV was significantly different from that of H3N2 viruses in recent previous TIVs. A significant rise in A/Switzerland/9715293/2013-specific Abs that induced degranulation (CD107a expression) of NK-92 cells was observed after vaccination in both HIV-negative (*P* = 0.0033) and HIV-positive (*P* < 0.0001) subjects (Fig. 5B). Consistent with the FcγRIIIa dimer-binding assay, we observed lower A/Switzerland/9715293/2013-specific NK-92 cell-activating Abs at baseline in the HIV-positive group; however, this did not reach statistical significance (*P* = 0.11). However, a strong positive correlation was observed between NK-92 activation ac-

tivity and FcγRIIIa-V158-binding activity (*r* = 0.78, *P* < 0.001; Fig. 5C).

A/Switzerland/9715293/2013 is a recent H3N2 antigenic variant. However, cross-reactive Abs able to mediate ADCC against immunologically novel influenza virus strains have been previously reported (26, 27). We therefore analyzed NK cell activation by serum Abs recognizing A/Switzerland/9715293/2013 in the presence or absence of baseline HI activity to this strain (Fig. 5D). Both prior to and after vaccination, we observed NK cell-activating Abs independently of baseline HI activity against A/South Australia/55/2014 [an A/Switzerland/9715293/2013 (H3N2)-like virus]. These results are consistent with previous work reflecting

FIG 4 FcγRIIIa dimer-binding activity to vaccine and nonvaccine influenza virus strains. (A) FcγRIIIa-V158-binding Ab responses (left panel) and FcγRIIIa-F158-binding Ab responses (center panel) to the 2015 TIV vaccine-HAs [A/Switzerland/9715293/2013 (H3N2), A/California/7/2009 (H1N1), and B/Phuket/3073/2013] and FcγRIIIa-V158-binding Abs to three nonvaccine HAs (A/Perth/16/2009 (H3N2), A/New Caledonia/20/1999 (H1N1), and B/Florida/4/2006; right panel). Responses are with background subtracted (SIV gp120-coated wells) and normalized to 5 μg/ml of IVIG. Plasma was diluted 20 times before analysis (40 times before analysis with A/New Caledonia/20/1999). Baseline and postvaccine data were compared using Wilcoxon matched-pairs test. HIV-negative and HIV-positive groups were compared using the Mann-Whitney test. *P* values of <0.05 were considered significant. Lines and error bars indicate means and SEM, respectively. (B) Correlation of the HA-specific Abs binding to the high-affinity (V158) and low-affinity (F158) FcγRIIIa dimer, analyzed with nonparametric Spearman correlation. Data shown for all three vaccine HAs, i.e., A/South Australia/55/2014, A/California/7/2009, and B/Phuket/3073/2013. Results for all subjects (baseline and postvaccine) are shown in the graph. (C) Correlation between HI titers and FcγRIIIa-V158 dimer-binding Abs to the three vaccine antigens, A/Switzerland/9715293/2013 (A/South Australia/55/2014 used in HI assay), A/California/7/2009, and B/Phuket/3073/2013, analyzed with the nonparametric Spearman correlation. Results for all HIV-negative and HIV-positive subjects (baseline and postvaccine) are shown in the graph.

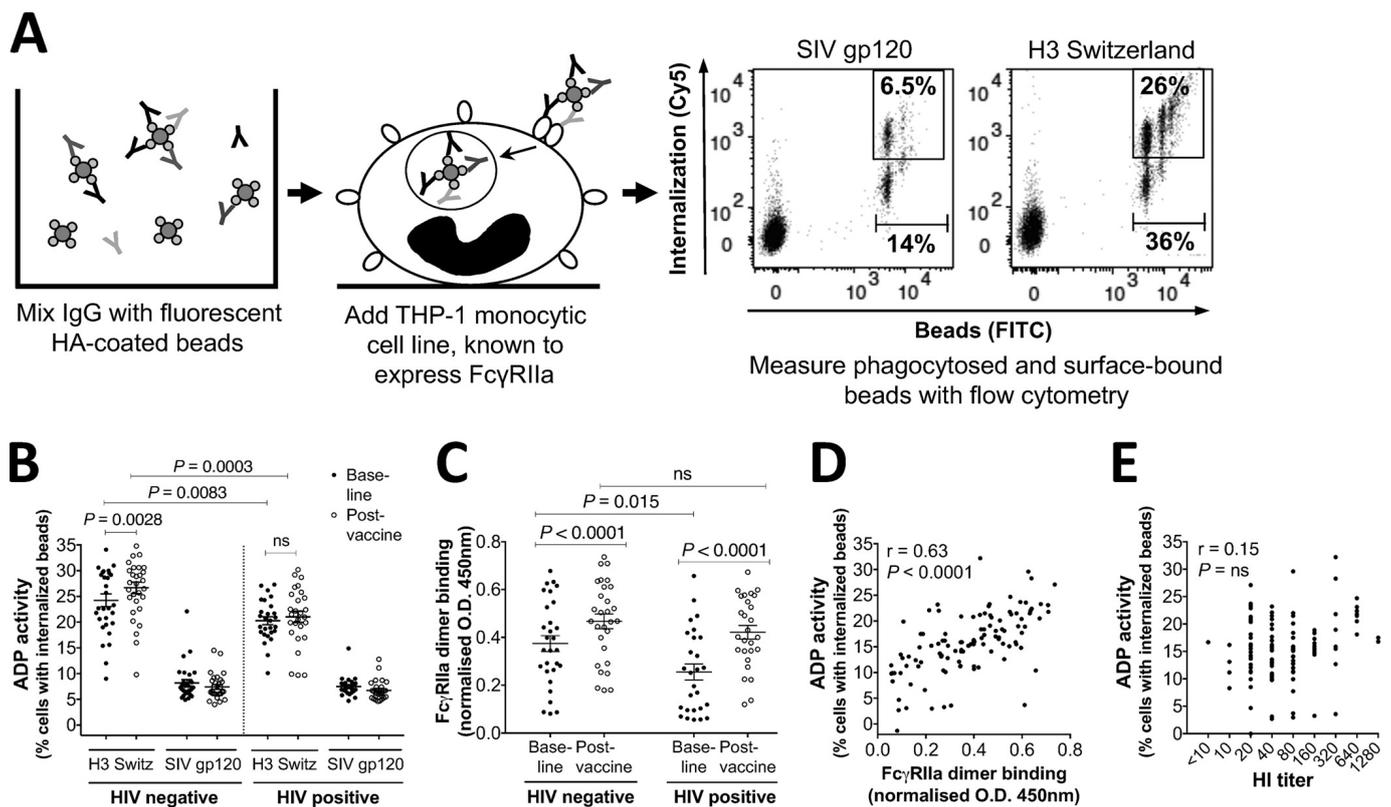


FIG 6 ADP assay with THP-1 monocytic cell line. (A) Assay setup. Fluorescently labeled beads were coated with both biotinylated HA from A/Switzerland/9715293/2013 (H3N2) and a fluorescent internalization probe and then opsonized with Abs and incubated with a THP-1 monocytic cell line, known to express Fc γ RIIa. Fluorescence derived from internalization probes on surface-bound beads was quenched with a complementary quencher probe before internalization was measured with flow cytometry. SIV gp120 protein was included in each experiment as a negative control. (B) ADP Ab responses to HA from A/Switzerland/9715293/2013 and to SIV gp120 protein in HIV-negative and HIV-positive subjects. ADP activity is measured as the percentage of monocytes with internalized beads. Baseline and postvaccine data were compared using the Wilcoxon matched-pair test. HIV-negative and HIV-positive groups were compared using the Mann-Whitney test. P values of <0.05 were considered significant. Lines and error bars indicate means and SEM, respectively. (C) Fc γ RIIa-H131-binding Ab responses to A/Switzerland/9715293/2013 (H3N2) HA. Responses are with background subtracted (SIV gp120-coated wells) and normalized to 5 μ g/ml of IVIG. Plasma was diluted 20 times before analysis. Baseline and postvaccine data were compared using the Wilcoxon matched-pair test. HIV-negative and HIV-positive groups were compared using the Mann-Whitney test. P values of <0.05 were considered significant. Lines and error bars indicate means and SEM, respectively. (D) Correlation between Fc γ RIIa-H131-binding Abs and ADP-stimulating Abs to HA from A/Switzerland/9715293/2013, analyzed with nonparametric Spearman correlation. Background-subtracted data are presented in the graph. Results for all HIV-negative and HIV-positive subjects (baseline and postvaccine) are shown in the graph. (E) Correlation between HI Ab titers and ADP activity to A/Switzerland/9715293/2013 (A/South Australia/55/2014 used in HI assay), analyzed with the nonparametric Spearman correlation. Background-subtracted ADP data are presented in the graph. Results for all HIV-negative and HIV-positive subjects (baseline and postvaccine) are shown in the graph.

the broader recognition of HA antigens by functional non-NAbs compared to NAbs (12, 26, 28).

Induction of HA-specific antibody-dependent phagocytosis by TIV. Fc-FcR interactions can mediate antiviral functions other than ADCC. ADP is mediated by Fc γ RIIa and may play a role in the prevention of viral infections such as HIV (29). To investigate if HIV infection alters the ADP Ab response to TIV vaccination, we assessed the ability of A/Switzerland/9715293/2013-specific Abs from HIV-negative and HIV-positive subjects to mediate phagocytosis of HA-coated beads in a THP-1 monocytic cell line that expresses Fc γ RIIa (Fig. 6A and B). We also assessed the Fc γ RIIa-H131-binding Ab response to A/Switzerland/9715293/2013 HA using a Fc γ RIIa-H131 dimer-binding assay (Fig. 6C). We detected significantly reduced ADP activity (percentage of THP-1 cells with internalized beads) in the HIV-positive group at baseline compared to the HIV-negative group ($P = 0.0083$; Fig. 6B). This was consistent with an observed reduction in baseline Fc γ RIIa-binding Abs in the HIV-positive group ($P = 0.015$;

Fig. 6C). ADP activity increased in response to TIV in both cohorts; however, significantly lower ADP activity was observed in the HIV-positive group after vaccination than in the HIV-negative group ($P = 0.0003$; Fig. 6B). However, this was not reflected in the Fc γ RIIa-binding assay, in which no significant difference in Fc γ RIIa-H131-binding Abs between the HIV-negative and HIV-positive subjects was observed after vaccination (Fig. 6C). There was a significant correlation between the ADP-stimulating Abs in the functional assay and Fc γ RIIa-H131-binding Abs ($r = 0.63$, $P < 0.001$) to the novel HA from A/Switzerland/9715293/2013 (Fig. 6D). Furthermore, we observed that subjects with high serological HI activity generally had high ADP activity (Fig. 6E), although this observation failed to reach statistical significance.

Correlations between CD4 T cell counts, duration of HIV infection, and Ab responses to TIV. The characteristic depletion of CD4 T cells in untreated HIV infection has been associated with reduced Ab responses to TIV (18, 30). We therefore examined in HIV-positive subjects any relationship between baseline CD4 T

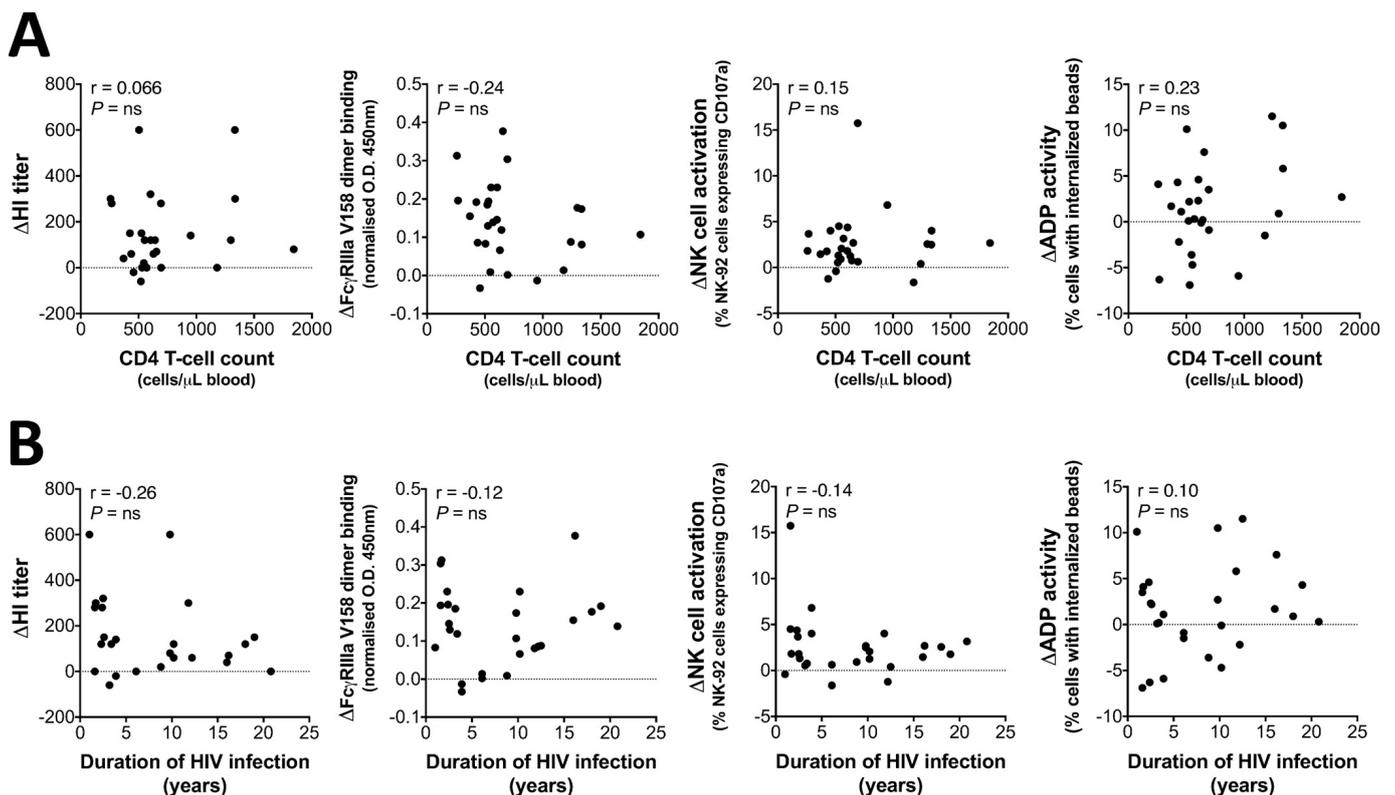


FIG 7 Correlations of CD4 T cell counts and HIV infection duration with functional Ab responses. (A) Correlation between CD4 T cell counts and the change in HI titer, Fc γ RIIIa-V158-dimer binding, NK cell activation, and ADP activity to A/Switzerland/9715293/2013 (postvaccine minus baseline response). (B) Correlation between the duration of HIV infection and the change in HI titer, Fc γ RIIIa-V158-dimer binding, NK cell activation, and ADP activity to A/Switzerland/9715293/2013 (postvaccine minus baseline response). Results for all 27 HIV-positive subjects are shown in the graphs. Analysis was by nonparametric Spearman's correlation.

cell counts (Fig. 7A) or the duration of HIV infection (Fig. 7B) and changes in HI activity, Fc γ RIIIa-V158-dimer binding, NK cell activation, and ADP activity to A/Switzerland/9715293/2013 following vaccination. No significant correlations were observed. These results likely reflect adequate treatment responsiveness to ART within our patient cohort, with generally high CD4 T cell counts observed in most individuals (mean, 729; range, 257 to 1,843).

DISCUSSION

NABs clearly mediate protective immunity to influenza (31, 32); however, recent studies have suggested that Abs with Fc-mediated functions play an important additive role in protective immunity (11). We recruited subjects with and without underlying HIV infection to receive the 2015 TIV and studied functional Ab responses prior to and after vaccination. At baseline, HIV infection was associated with reduced Fc-mediated Ab functionality, despite similar serological HI activity to HIV-negative subjects. Although the reason for more-severe influenza infections in HIV-positive subjects is almost certainly multifactorial (20, 33–35), our results suggest that a reduction in Abs able to effectively interact with FcR may contribute to this increased risk. We detected NK cell-activating Abs to HA from the novel circulating influenza virus strain A/Switzerland/9715293/2013 in the majority (93%) of both HIV-negative and HIV-positive subjects in the absence of HI responses to this strain. This is consistent with previous studies showing that previous influenza exposures can elicit functional Abs that can cross-react with other influenza virus strains (26, 28).

Indeed, highly cross-reactive, ADCC-mediating Abs are present in humans even to avian H5 and H7 in the absence of prior exposure (26, 28). Studies in both mice and macaques have shown a potential role for cross-reactive non-NABs induced by prior seasonal influenza infections in the protection from pandemic influenza exposure (12, 36). These cross-reactive ADCC-mediating Abs can bind more conserved regions of the influenza HA, such as the stem (11, 26).

TIV is a partially effective strategy to prevent influenza, but its efficacy wanes in those in greatest need. Poor HI responses to TIV in HIV-positive subjects have been reported, but most studies analyzed cohorts prior to the widespread use of ART (18, 21).

In this study, we found that HI Abs to vaccine influenza virus strains increased equally after vaccination in the HIV-negative and HIV-positive cohorts. Furthermore, despite lower baseline levels of functional Abs in the HIV-positive group, both groups responded well to vaccination. Indeed, across several assays, the net increase in functional Ab responses to influenza increased to a greater extent in the HIV-positive group (albeit starting from a lower baseline level) than the HIV-negative group. These results are consistent with the use of ART, the generally high CD4 T cell count, and the undetectable HIV load in almost all subjects (Table 1). Indeed, the relative immunocompetence of our HIV-infected cohort precluded any significant correlations with responses to vaccination. Taken together, our results both support the use of ART in HIV-infected subjects to improve anti-influenza immu-

nity and support the use of TIV in HIV-infected subjects in the current ART era since our cohort responded adequately to vaccination.

We acknowledge that the physiological relevance and protective role of ADCC and ADP Abs in anti-influenza immunity in humans require further study. Studies in mice and macaques suggest that ADCC Abs may assist in protection against or clearance of influenza virus infections (11, 12). Supporting a role for ADCC, Jegaskanda et al. found that the elderly had enhanced ADCC responses to the pandemic H1N1/09 virus strain prior to the epidemic. This presumably reflected distant prior infections with more closely related H1N1 strains and may contribute to reduced disease incidence within the elderly population during the 2009 pandemic (37). Ongoing studies such as the passive transfer of polyclonal immune serum in severe influenza infections (Clinical Trials registration no. NCT02287467) may help tease out a role for non-NAbs in control of human influenza infections.

We recognize that there are some limitations to our study. FcR-binding capacity and Ab-dependent NK cell activation are useful as surrogate markers of ADCC activity, but they do not directly quantitate Ab-mediated cell killing. We have, however, previously shown that Ab-mediated clearance of influenza virus-infected cells can be measured concurrently with NK cell activation (26). Further, we recently developed methods to study the Ab-mediated uptake of opsonized influenza virions, which in a separate study of influenza-specific immunity correlated well ($r = 0.80$, $P = 0.0029$) with the ADP assay employed here (43). Thus, we are confident that the assays we used have functional significance.

Our results suggest that HIV-infected subjects could derive significant benefit from influenza vaccination. However, the reported NK cell exhaustion and dysfunction in HIV-infected subjects (38–41), which only partially normalize after ART, may reduce the effectiveness of Abs with Fc-mediated functions. Further investigation into the FcγRIIIa surface expression on autologous NK cells and the TIV-induced autologous NK cell activation in HIV-infected subjects is warranted to help us better understand the effect of HIV infection on NK cell-mediated ADCC after influenza vaccination.

A number of further studies are suggested by this work. Although we observed increased anti-influenza immunity to non-vaccine strains after vaccination, further experiments with more-divergent influenza virus strains are required to assess the full breadth of TIV-induced functional non-NAb responses. The role of vaccination in boosting preexisting versus generating new non-NAb responses remains to be clarified. Technologies to define influenza-specific B cells and sequence their Ig genes allow a more detailed probe of preexisting versus new influenza-specific Ab responses and could be applied to non-NAb responses (42).

In conclusion, this study suggests that HIV-infected individuals have diminished functional Ab responses but that these can be restored with seasonal influenza vaccination. This highlights the importance of annual influenza vaccination within this at-risk group. Further investigation into the functional importance of Fc-mediated Ab immunity to influenza is warranted.

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