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Increased influenza-specific antibody avidity in HIV-infected females compared to HIV-infected males on antiretroviral therapy

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Abstract

It is recommended that HIV-infected individuals receive annual influenza vaccination due to their high susceptibility to influenza infection, especially among females. However, there have been few studies investigating sex-related responses to influenza vaccine in antiretroviral (ART)-treated HIV-infected subjects. In this study, 26 aviremic ART-treated HIV-infected subjects and 16 healthy controls were enrolled in the current study. Blood was collected prior to vaccination (D0), on days 7-10 (D7), and on days 14-21 (D14) following administration of the 2013-2014 seasonal influenza vaccine. A series of analyses evaluated the serological and cellular responses following influenza

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vaccination. Female HIV-infected subjects had increased influenza-specific antibody avidity relative to male HIV-infected subjects, but similar plasma levels of influenza-specific binding antibodies and neutralizing antibodies. Increased cycling B cells and follicular helper CD4+ T (Tfh) cells were observed in female HIV-infected subjects pre- and post-vaccination compared to male HIV-infected subjects, and cycling Tfh cells were directly correlated with influenza-specific antibody avidity. Moreover, plasma testosterone levels were inversely correlated with antibody avidity index. The magnitude of microbial translocation (plasma lipopolysaccharide (LPS)) level was directly correlated with influenza-specific antibody avidity. Circulating 16S rDNA microbiome showed that enrichment of specific species within *Proteobacteria* was associated with influenza-specific antibody avidity. These results, including differences based on sex and correlations, were only observed in HIV+ subjects but not in the healthy controls. This study demonstrated sex differences in influenza-specific antibody avidity in ART-treated HIV disease, and provides valuable information on vaccination strategy in the ART-treated HIV-infected population.

Keywords

HIV; influenza vaccine; antibody avidity; antiretroviral therapy

INTRODUCTION

HIV-1 infection is associated with an elevated incidence of influenza infection especially in females (1). Seasonal influenza vaccination is highly recommended for all HIV-infected individuals (2) due to its ability to prevent influenza infection and reduce influenza-related morbidity and mortality (3). It is unclear, however, whether there is a difference in the immune response to the influenza vaccine between males and females in this patient population.

Sex differences in immune responses against infectious diseases and vaccines have been shown among various populations in previous studies (4). In general, males are more susceptible and experience more severe infections caused by parasites, fungi, bacteria, and viruses due to their weak immune responses to these pathogens compared to females (5). Moreover, many studies in healthy populations have found that females develop more robust antibody responses to vaccination than males (6–8). However, females also develop higher inflammatory responses to vaccination which result in increased adverse biological reactions to vaccination compared to males (9).

During the 2009 H1N1 pandemic, females showed a higher mortality rate after exposure to the pathogenic influenza A virus compared with men (10). This paradoxical effect may be related to the development of immune-mediated pathologies during influenza infection that occur more frequently in females (11). The increased risk of autoimmune diseases in women further support this hypothesis (12).

The purpose of the current study was to evaluate the difference in antibody response for males versus females in both HIV-infected subjects and healthy controls and characterize and link T and B cellular responses to serologic antibody responses. Understanding the

immune perturbations in HIV can help improve the immunogenicity of vaccine regimens and establish guidelines to improve immune responses in this vulnerable population.

RESULTS

Study population

Twenty-six HIV-infected subjects (9 females, 17 males) and 16 healthy controls (11 females, 5 males) were included in the current study. The clinical characteristics of the two groups are shown in Table 1.

Increased influenza-specific antibody avidity and hemagglutination (HA) inhibition titers in females compared to males in antiretroviral-treated HIV infection

Serologic influenza-specific IgA, IgG, and IgM titers were evaluated by enzyme-linked immunosorbent assay (ELISA). Antigen-specific IgG, IgA, and IgM increased following vaccination in both HIV-infected subjects and controls with no statistically significant differences based on sex (Fig. 1A). However, there was a trend toward higher vaccine-induced fold-changes of antigen-specific IgG and IgM on day 14 after vaccination (D14) compared to day 0 prior to vaccination (D0) in HIV-infected females compared to males ($P = 0.215$, $P = 0.625$, respectively) (Fig. 1B).

We then performed viral micro-neutralization assays to detect the plasma titers of neutralizing antibodies which are considered protective in influenza viral infection (13). Consistently, seasonal influenza vaccination significantly induced neutralizing antibodies (NAb) compared to baseline levels against H1N1, B, and H3N2 strains in both HIV-infected subjects ($P < 0.0001$, $P = 0.023$, $P < 0.0001$, respectively) and healthy controls ($P = 0.003$, $P = 0.0005$, $P = 0.044$, respectively), but there were no statistically significant differences between the sexes observed in either group (Fig. 1C and 1D).

The somatic hypermutation (SHM) of the immunoglobulin variable gene after vaccination allows for generation of high-affinity antibodies (14). Therefore, in addition to neutralizing antibodies, the avidity of an antigen-specific antibody is thought to be an important factor in infection and disease (15). To define the avidity of vaccine-specific antibodies, the guanidine hydrochloride (GuHCl)-modified ELISA method was applied (16). Four concentrations of GuHCl (0.5, 1.0, 1.8 and 2.5 M) were tested. The avidity index value was determined by plotting optical density (OD) values against the four serum dilution factors. Two lines were plotted, one representing samples treated with GuHCl (condition-1) and one representing samples without any treatment (condition-2). This avidity index was calculated by the ratio of condition-1 to condition-2. Notably, HIV-infected females had increased IgG avidity index compared to HIV-infected males (Fig. 1E), a difference that was not observed in the healthy control group (Fig. 1E). These results suggest that HIV-infected females develop higher IgG avidity compared to HIV-infected males after receiving multiple seasonal influenza vaccines.

The plasma testosterone level correlation with antibody avidity index

A previous study showed that hormones are one of the most important mediators of sex differences in immune responses (4). Thus, we investigated the relationship between hormone levels and avidity index. We measured testosterone, progesterone, and 17β -estrogen in all subjects at D0. As expected, males from both the HIV-infected and healthy control groups had higher levels of testosterone compared to females. Interestingly, however, there were no statistically significant differences in 17β -estrogen and progesterone levels in males vs. females for either group (Fig. 2A–2C). We then investigated the association between hormone levels and antibody avidity. Testosterone was inversely correlated with avidity index both at D0 and D14 in the HIV-infected subjects (Fig. 2D and 2E) but not in the healthy controls (D0: $r = 0.14$, $P = 0.60$; D14: $r = 0.28$, $P = 0.30$). There were no significant correlations between progesterone or 17β -estrogen with antibody avidity for either group (data not shown).

Increased cellular response in HIV-infected females compared to HIV-infected males and its correlation with antibody avidity index

To determine the dynamics and magnitude of cellular responses elicited by vaccination, we analyzed the frequency of influenza IgG+ antibody-secreting cells (ASCs) and cycling of memory B cells pre- and post-vaccination. Influenza IgG+ ASCs were quantified by enzyme-linked immunospot (EliSpot) among sorted B cells (purity > 97%) after culturing with vaccine antigens without stimulation, cytokines or other agonists. B cells activated by antigen are capable of rapid proliferation and differentiation into short-lived plasmablasts (17). Because the peak transient responses of plasmablasts to the influenza vaccine occur in a short time period (18), 7 days after vaccination (D7) was chosen as the collection time point. To determine changes in IgG+ ASC frequency post-vaccination, the baseline frequency of IgG+ ASCs on D0 was subtracted from D7. Consistent with the serologic antibody result, the ASCs were dramatically increased after vaccination in all subjects from both groups regardless of sex (Fig. 3A).

Next, the frequency and cycling of memory B cells were assessed by flow cytometry. The frequency of memory B cells in peripheral blood mononuclear cells (PBMCs) was similar in males and females for both HIV-infected subjects and healthy controls (Fig. 3B). The expression of Ki-67, a marker of proliferation and cycling in memory B cells (19, 20), was elevated in HIV-infected females compared to everyone else (HIV-infected males and healthy male and female controls) both pre- and post-vaccination (Fig. 3C). These results suggest increased activation of B cells in HIV-infected females compared to HIV-infected males and healthy controls regardless of vaccination status.

Follicular helper CD4+ T (Tfh) cells are the specialized providers for B cells in the germinal center (GC) that assist in the development of SHM in ASCs and antibody affinity maturation (21, 22). Therefore, we analyzed the frequency of Tfh cells in total CD4+ T cells in males and females on D0 and D7 (Fig. 3D). Similar to B cell frequencies, there was no sex-based difference in the frequencies of Tfh cells at baseline or post-vaccination for either group. Next, we assessed Tfh cell cycling, which is important for SHM and selection of GC B cells (21). A higher percentage Ki-67 expression in Tfh cells was seen in HIV-infected females on

D7 after vaccination when compared to baseline, but not in HIV-infected males and healthy females and male controls (Fig. 3E). Although HIV-infected females had higher Ki-67+ Tfh cells compared to HIV-infected males at D7 (Fig. 3E), there was no sex difference in the healthy controls, consistent with our observed B cell response.

To determine if heightened Ki-67 expression in Tfh cells was related to high avidity index in HIV, we investigated the relationship between Ki-67+ Tfh cells and the avidity of serologic antibodies with both at D0 and then for Ki-67+ Tfh cells at D7 and the avidity of serologic antibodies at D7. We observed a positive correlation in both analyses between the frequency of cycling Tfh cells and the antibody avidity (Fig. 3F–3G). These significant correlations were not observed among the healthy controls (data not shown).

Lipopolysaccharide (LPS) and the plasma microbiome are related to the antibody avidity index in HIV-infected subjects but not in healthy controls

Toll-like receptors (TLRs) play an important role in antibody production, as TLR signaling is necessary for dendritic cell maturation, B cell activation, and GC formation (23, 24). Therefore, we investigated whether the difference in the baseline level of systemic TLR agonists (*e.g.*, LPS) was associated with vaccine antibody responses. Interestingly, plasma LPS levels tended to be higher in HIV-infected females relative to HIV-infected males, although the difference did not reach statistical significance (Fig. 4A). Moreover, plasma LPS levels at baseline were positively correlated with both D0 and D14 antibody avidity after vaccination in HIV-infected subjects (Fig. 4B–4C), but neither of these relationships were significant among the healthy controls (data not shown).

To investigate TLR signaling pathway response to persistent LPS stimulation *in vivo*, we purified B cells at baseline and assessed mRNA relative expression of the *MyD88* gene, an adaptor protein required for TLR signaling. Consistently, *MyD88* expression at D0 tended to be higher in the HIV-infected females relative to the HIV-infected males, although it did not achieve statistical significance (Fig. 4D). Furthermore, there was a direct correlation between the *MyD88* expression and the antibody avidity index at D0 of vaccination in HIV-infected subjects (Fig. 4E), but not in healthy controls (data not shown). The results indicate that the TLRs/*MyD88* pathway in B cells may be involved in antibody affinity maturation in ART-treated HIV-infected patients.

Studies of the microbiome have shown that distinct microbial species have different effects on the host immune system (25). Since the quantity of baseline systemic microbial translocation was associated with the antibody avidity (Fig. 4B), we wanted to know whether the diversity of the translocated microbial species would affect the avidity of antibody. Using bacterial 16S rDNA extracted from plasma, we identified microbial products via bacterial taxonomy through microbiome analysis. To control for potential 16S rDNA contamination from the reagents (26), we removed genera from experimental samples at the operational taxonomic unit (OTU) level if they were detected in the water controls. To compare overall microbial community diversity, the Gini Simpson diversity index was applied. The diversity of microbiota composition did not differ based on sex either in the HIV-infected group ($P = 0.907$) or in the healthy control ($P = 0.317$) (Fig. 4F).

Next, we assessed the composition of plasma microbial communities within females and males in both the HIV-infected subjects and healthy controls. The predominant phylum-level products were similar in both HIV-infected subjects and controls with no differences based on sex. In HIV-infected females, predominant plasma phylum-level microbial products in order of decreasing prevalence were from *Proteobacteria* (% mean $\pm$ standard deviation (SD), 53.9% $\pm$ 20.8%), *Firmicutes* (21.0% $\pm$ 22.8%), and *Actinobacteria* (7.3% $\pm$ 8.9%), and, in HIV-infected males, the predominant products were from *Proteobacteria* (62.7% $\pm$ 20.2%), *Firmicutes* (16.8% $\pm$ 12.1%), and *Actinobacteria* (10.1% $\pm$ 10.1%). There was a lower ratio of *Firmicutes/Proteobacteria* in HIV-infected males compared to HIV-infected females (0.27 versus 0.39 respectively). In female controls, the predominant products were from *Proteobacteria* (49.1% $\pm$ 22.9%), *Firmicutes* (20.0% $\pm$ 19.7%), and *Actinobacteria* (18.0% $\pm$ 21.1%). In male controls, the predominant phylum-level products were from *Proteobacteria* (31.7% $\pm$ 22.0%), *Firmicutes* (27.6% $\pm$ 16.4%), and *Actinobacteria* (19.3% $\pm$ 19.4%) (Fig. 4G). In healthy controls, the ratios of *Firmicutes/Proteobacteria* in males compared to females were 0.87 versus 0.41 respectively. Therefore, healthy controls had greater ratio of *Firmicutes/Proteobacteria* in both sexes.

Next, we analyzed if any species shown as the taxa at the OTU level were correlated with the antibody avidity within the HIV-infected subjects. We found a significant inverse correlation between antibody avidity and particular species within the *Proteobacteria* and *Actinobacteria* phyla (Fig. 4H). We also found positive correlations between antibody avidity and particular species within the *Firmicutes* and *Proteobacteria* phyla. At the genus level, increased relative abundances of such as *Enterobacter* and *Klebsiella* were associated with decreased antibody avidity, while increased relative abundances of such as *Attoposipes*, *Comamonas*, and *Stenotrophomonas* were associated with increased antibody avidity in HIV-infected subjects (Fig. 4H).

DISCUSSION

HIV-infected patients are at an increased risk for influenza viral infections and are considered to be a high-priority group for influenza vaccination (27). However, impaired antibody responses to vaccination in ART-naïve HIV-infected subjects have been reported (28, 29). Importantly, our previous data showed that seasonal influenza vaccine serologic responses are comparable to healthy controls once HIV-infected patients have been successfully treated with ART (30). The aim of this current study was to determine whether there is a difference based on sex in the serologic and cellular responses induced by seasonal influenza vaccination in ART-treated virologically-suppressed HIV-infected subjects. Such a difference in response to vaccination based on sex has been shown previously in the general population, where females mount a more robust immune response compared to males (31–33). We found that the antibody avidity exhibited sex-based differences in the HIV-infected subjects, where females had an increased avidity index compared to males; this sex-based difference was not observed in the healthy control group. In addition, cycling of Tfh cells and memory B cells was increased in HIV-infected females compared to HIV-infected males, a finding again not observed among the healthy controls. On the other hand, neither the HIV-infected group nor healthy controls exhibited any sex-based differences in the frequency of memory B cells or Tfh cells.

We questioned whether the difference in antibody avidity could be attributed to a difference in the number of previous seasonal influenza vaccinations received between the two sexes. For some infections, such as HIV, antigen-specific antibodies acquire affinity development by continuous antigen stimulation (34). However, longitudinal tracking of vaccine-induced HA-specific clones have shown no overall increase in SHM over time (35). Therefore, repeated annual influenza immunization likely does not enhance the quality of influenza-specific antibody (36), making the HA-specific avidity difference that we observed between male and female HIV-infected subjects unlikely to be attributable to a difference in the number of previous vaccinations. Nonetheless, there was no sex difference in the frequencies of previous seasonal influenza vaccinations in HIV-infected subjects (Table 1).

We also observed a difference in Tfh cell cycling expression in HIV-infected males and females, which was not seen among the healthy controls, but there was no difference in the number of cells for either group. Tfh cells are specialized providers of B cell help and are essential for generating a protective and high-affinity humoral response to antigen stimulation (37, 38). The selective survival and expansion of antigen-specific GC B cell clones carrying somatic mutations account for the progressive increase in antibody affinity after vaccination (22). Thus, the higher cycling expression in Tfh cells and memory B cells in HIV-infected females compared with males in our study indicates that Tfh cell activation may contribute to the antibody avidity difference observed between the sexes.

Interestingly, testosterone was inversely correlated with antibody avidity index in our study for the HIV-infected group (a correlation not observed among the healthy controls), but there was no association with influenza-specific NAb or binding antibodies. Our data is in line with other studies that testosterone provides an immunosuppressive function. Testosterone downregulates the expression of TLR4 on monocytes and macrophages, causing a diminished LPS-induced inflammatory cytokine response (39), which ultimately leads to a decrease in T and B cell proliferation (40).

In addition, plasma LPS levels at baseline were positively correlated with the antibody avidity in HIV-infected subjects, a correlation again not observed among the healthy controls. Our previous data showed that the plasma level of TLR4 ligand LPS was associated with inflammation and B cell activation in HIV disease (41). Furthermore, high-affinity influenza-specific antibodies driven by TLRs have been found in systemic lupus erythematosus patients in response to vaccination (42). As the receptor for LPS, TLR4 signaling in B cells has been reported to increase B cell migration and accumulation in GC dark zones, perhaps helping GC reactions (43). Knockout of TLR4 or MyD88, one of the adaptor proteins required for signaling from TLR4, results in a reduced percentage of GC B cells compared with wild-type B cells (44), suggesting that the TLR4/MyD88 signaling pathway may help B cell retention and development in the GC. Consistent with our observed positive correlation between LPS and antibody avidity among the HIV-infected group, we also identified a similar correlation in our study between LPS and the mRNA level of *MyD88* gene expression in B cells in the HIV-infected group but not within the healthy controls. These results suggest that microbial translocation and/or TLR signaling may play a role in the increased antibody avidity in ART-treated virologically-suppressed HIV-infected females. Moreover, our plasma microbiome analysis showed that a lower ratio of *Firmicutes*/

Proteobacteria in HIV-infected individuals relative to healthy controls regardless of sex, which is consistent with gut microbiome found in HIV patients (45). In addition, a particular microbiome was associated with antibody avidity in HIV-infected subjects.

We found that increased relative abundance of *Enterobacter cloacae* and *Klebsiella spp.* was associated with decreased antibody avidity in HIV-infected subjects. Previously studies showed that *Enterobacter cloacae* was invasive to epithelial cells (46) and induced lysis of the murine macrophage J774 cell line (47). These findings suggest that *Enterobacter cloacae* may adhere to and penetrate epithelial cells, induce apoptosis, and affect antigen phagocytosis and presentation. In general, B cell proliferation and germline mutation in the GC are necessary for antibody affinity mutation (22). Notably, *Klebsiella pneumoniae*, one of *Klebsiella spp.*, has been shown to stimulate human B cells to mature into plaque forming cells without prior B cell proliferation (48). In future, we will identify the effects of these bacteria on host immunities, as this has a great therapeutic potential.

Previously studies showed that females had increased antibody responses following influenza virus vaccination in the general population, including influenza hemagglutinin inhibition and neutralizing antibody titers against H1N1, H3N2, and influenza B antigens (33, 49). However, we did not observe any gender differences in the cellular and serologic responses to the influenza vaccine within our healthy control group. This may be due to the small sample size in the control group which limited our ability to detect gender differences. In addition, the frequencies of previous vaccination in healthy controls is not available in the current study, which could be responsible for some gender differences in controls and patients in the current study and gender differences in healthy controls from previous studies and our study (50). However, our findings within the HIV-infected group are consistent with the immunological changes seen in chronic HIV infection and shed light on the unique aspects and sex-based differences in the immune response to influenza vaccine in this vulnerable population.

It is unclear, however, if increased influenza-specific antibody avidity in HIV-infected females represents increased degree of protection from influenza infection. As shown in the general population, it may represent a stronger vaccine responsiveness in HIV-infected women compared to HIV-infected men. However, females have been shown an increased risk of severe influenza-associated complications compared to males (51), and increased titer and avidity of influenza-specific non-NAb after influenza virus infection were associated with clinical severity and poorer outcomes in the general population (15). Furthermore, animals with high avidity anti-HA2 antibodies had increased H1N1 infection through promoting viral membrane fusion compared to animals with low avidity antibody responses (52). In the current study, antibody avidity is independent of the titers of protective influenza-specific antibodies generated in response to vaccination in HIV+ subjects, suggesting that antibody avidity may not associate with protections against influenza infection. Future studies on the clinical significance of increased avidity antibody responses in HIV-infected women may provide information on vaccination strategies in ART-treated HIV-infected patients.

MATERIALS AND METHODS

Study design/population

Twenty-six HIV-infected subjects and 16 healthy controls were included in the current analysis. Inclusion criteria for the HIV-infected subjects included current use of combination ART for ≥ 2 years and a plasma HIV-1 RNA < 50 copies/mL. All subjects had received the influenza vaccine in the previous year and were enrolled prior to receiving the 2013–2014 influenza vaccine. The day of vaccine administration was designated as D0. Blood was collected on D0 prior to vaccination and then on days 7–10 (D7) and days 14–21 (D14) post-vaccination. The study was approved by the institutional review board of the Medical University of South Carolina (Pro00020606); all subjects provided written informed consent.

Influenza virus strains and vaccination

The influenza virus strains for the 2013–2014 seasonal vaccine included two A subtypes [A/Christchurch/16/2010, NIB-74 (H1N1) (an A/California/7/2009-like virus); A/Texas/50/2012, NYMC X-223 (H3N2) (an A/Victoria/361/2011-like virus)] and one B subtype (B/Massachusetts/2/2012). Subjects received a single intramuscular dose of licensed inactivated trivalent vaccine from a single lot (Fluvirin, Glaxo-Smith Kline), formulated to contain 15 µg of HA of each strain.

Flow cytometry

Whole blood was collected in EDTA-containing tubes for immediate plasma isolation and cryopreservation at –80°C without prior thawing until analysis. Peripheral blood mononuclear cells were isolated from the same tubes of whole blood over a Ficoll-Paque, and used for annexin V assays. Except for the annexin V assays, all other assays, such as Ki-67, the percentage of B and Tfh cells, were done using fresh whole blood samples. For surface staining, antibodies were incubated with either whole blood or PBMCs at room temperature for 15 minutes. After surface-staining of the whole blood, red cells were lysed, and the cells were washed and analyzed by flow cytometry.

For Ki-67 staining, the Foxp3 buffer sets (eBioscience, San Diego, CA) were used according to the manufacturer's protocol. The fluorochrome-labeled monoclonal antibodies used in this study included: anti-human CD3 (OKT3), anti-human CD4 (RPA-T4), anti-human CD8 (RPA-T8), anti-human CD27 (M-T271), anti-human CD45RA (HI100), anti-human CD38 (HIT2), anti-human IgD (IA6-2), anti-human CD19 (HIB19), anti-human CXCR5 (RF8B2), anti-human CXCR3 (G025H7), anti-human PD1 (MIH4), anti-human Ki-67 (Ki-67), annexin V (BD Pharmingen), and isotype control Abs (BD Pharmingen). Cells without annexin V staining were used as controls for the apoptosis gating strategy. All others were gated based on isotypes. Cells were collected in a Guava 8HT flow cytometer (Millipore, Billerica, MA), and data were analyzed by FlowJo software (Version 10.0.8).

Enzyme-linked immunosorbent assay for influenza-specific antibody

Microtiter plates were coated with a 1:20 dilution of the 2013–2014 seasonal influenza vaccine. Diluted plasma was added to each well for 1 hour at room temperature. After washing, biotin-labeled goat anti-human IgG, IgM, or IgA (KPL, Gaithersburg, MD) was

added to the microtiter wells and cultured for 60 minutes. Horseradish peroxidase-conjugated streptavidin (HRP-streptavidin) and 2,2'-Azino-di (3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate solution were used to detect binding. Absorbance was measured at 405 nm; antibody concentrations were calculated in duplicates using a standard curve (12.5–1000 pg/mL).

Enzyme-linked immunosorbent assay for hormone detection

Plasma testosterone, progesterone, and 17 β -estrogen levels were measured by human hormone ELISA kit (Abcam, Cambridge, UK) according to the manufacturer's protocol.

Microneutralization assay

This method is described in detail in our previous study (30). Briefly, plasma samples were serially-diluted and heat-inactivated. Plasma samples were mixed with tissue culture-infective doses (TCID₅₀) for the H1N1 virus. The cells were cultured, and the presence of viral protein was detected by ELISA. The endpoint titer was calculated by the reciprocal of the highest dilution of plasma with an OD value less than X, where $X = [(average\ of\ V\ wells) - (average\ of\ C\ wells)]/2 + (average\ of\ C\ wells)$.

Antibody avidity index

The modified avidity index detection was performed by ELISA-based methods (16). Briefly, plates were initially coated with a 1:20 dilution of the 2013-2014 seasonal influenza vaccine, and plasma samples were diluted to yield an optical density of 1.0 ± 0.2 . One hundred μ L of the diluted plasma was added to each well for 1 hour with shaking at room temperature. After washing, 100 μ L of 0.5-2.5 M GuHCl was added for 15 minutes, and buffer without GuHCl was added to set as a control. Horseradish peroxidase conjugated-labeled goat anti-human IgG (KPL, Gaithersburg, MD) was added to the microtiter wells and cultured for 60 minutes. ABTS substrate solution was used to detect binding, and absorbance was measured at 405 nm. The avidity index was defined as the ratio of the area with GuHCl to the area without GuHCl.

Plasma lipopolysaccharide

Plasma samples were pretreated by dilution to 10% with endotoxin-free water and subsequent heating to 85°C for 15 minutes to inactivate inhibitory proteins. Plasma LPS levels were then measured by the endpoint chromogenic *Limulus* amebocyte lysate (LAL) assay kit (Lonza, Switzerland) according to the manufacturer's protocol. Background was subtracted, and LPS levels were calculated by the standard regression line.

Enzyme-linked immunospots for plasmablasts and memory B cells

The frequencies of influenza-specific IgG ASCs were measured by EliSpot assays as previously described (53). Briefly, plates were coated with the commercial 2013-2014 seasonal influenza vaccine (5 μ g/mL) and incubated overnight at 4°C. Total B cells were isolated from freshly-prepared PBMCs using a negative selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Freshly-isolated B cells (purity >95%) were cultured at the concentration of 0.5 million cells/well for 18-24 hours at 37°C, and the plates were

incubated with a biotinylated mouse anti-human IgG (Jackson ImmunoResearch, West Grove, PA) for 2 hours. Finally, the plates were incubated with alkaline phosphatase-conjugated streptavidin and developed with substrate. Spots were enumerated with an AID reader and software (San Diego, CA).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis of mRNA expression

The total RNA from B cells was extracted using the RNeasy Micro kit following the manufacturer's manual (Qiagen, Valencia, CA). Complementary DNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Gene expression analysis of *MyD88* was performed by pre-developed real-time PCR (*MyD88*, Hs01573837_g1) (Invitrogen). Gene expression levels were transformed to relative normalized expression, which is shown as $-\Delta\Delta Cq$ (Bio-Rad CFX Manager 3.1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control.

Plasma microbial DNA extraction, sequencing and data process

Microbial DNA was extracted from 400 μ L plasma and water control using QIAamp UCP Pathogen Mini kit (Qiagen) according to the manufacturer's instructions. The 16S rRNA gene V4 variable region PCR primers 515/806 with barcode on the forward primer were used in a 30 cycle PCR (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After checking by 2% agarose gel to determine the success of amplification and the relative intensity of bands, samples were purified using calibrated Ampure XP beads and then used to prepare the DNA library by following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX). Sequences were depleted of barcodes and primers, then short sequences <200 bp, sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6 bp were removed. Next, sequences were denoised and OTUs were defined clustering at 3% divergence (97% similarity), followed by removal of singleton sequences and chimeras. Final OTUs were taxonomically-classified using BLASTn against a curated database derived from National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDPII, East Lansing, MI) and compiled into each taxonomic level into both "counts" and "percentage" files. To remove potential bacterial 16S rDNA contamination from molecular biological reagents, we removed genera from experimental samples at the OTU-level if they were detected in the water controls. Operational taxonomic unit tables and different levels of taxonomy tables derived from the sequencing process described above were imported to R statistical software version 3.3.1 (The R Foundation for Statistical Computing, Vienna, Austria) for statistical analysis. Simpson index of diversity which measures the richness and evenness of a

microbiome community was calculated using the Vegan package (The R Foundation) to measure α diversity of each sample.

Statistical analysis

Conventional measurements of the central location and dispersion were performed to describe the data, and the differences in continuous measurements between the two groups were compared by the Wilcoxon matched-pairs signed rank test (paired) or Mann-Whitney's U test (unpaired). To explore associations between pairs of continuous variables, Spearman's correlation test was used. Comparison analysis was performed using SPSS software version 16.01 (Chicago, IL, USA). All tests were 2-sided, and $P < 0.05$ was used to denote statistical significance.

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Figure 1. Plasma levels of antigen-specific antibody, neutralizing antibody titers, and avidity index on day 0 and day 14 post-vaccination

(A) Median concentrations of vaccine antigen-specific IgG, IgA, and IgM in females (rhombus) and males (stars) on day 0 (D0) and day 14 (D14). (B) Antibody (Ab) fold-changes of vaccine antigen-specific antibody (D14 versus D0) in females (light) and males (dark) shown by box and whisker plots (median with interquartile range). (C) Median dilution of 50% protection by vaccine-neutralizing antibodies (NAb) against H1N1, H3N2, and B viruses in females (rhombus) and males (stars). (D) Fold-changes of neutralizing titers

(D14 versus D0) in females (light) and males (dark) shown by box and whisker plots (median with interquartile range). (E) Median levels of IgG, IgA, and IgM anti-influenza antibodies avidity in females (rhombus) and males (stars) on day 0 (D0) and day 14 (D14) post-vaccination.

Figure 2. Testosterone is inversely correlated with the antibody avidity in HIV-infected subjects but not in healthy controls

Median concentrations of testosterone (A), 17β -estrogen (B), and progesterone (C) from females (light) and males (dark) shown by box and whisker plots (median with interquartile range). (D) Correlation analysis between plasma levels of testosterone on day 0 (D0) and the avidity index of the influenza-specific IgG antibody on D0 in HIV-infected subjects. (E) Correlation analysis between plasma levels of testosterone on D0 and the avidity index of the influenza-specific antibody on day 14 (D14) in HIV-infected subjects. r = Spearman correlation coefficient.

Figure 3. Cellular immune responses to vaccination

(A) Increased antibody-secreting cells (ASC) from both males and females on day 7 (D7) measured by enzyme-linked immunospots. (B) Median frequencies of memory B cells (CD3-CD19+CD27+IgD-) among total B cells from females (rhombus) and males (stars). (C) Ki67 expression among memory B cells from females (light) and males (dark) shown by box and whisker plots (median with interquartile range). (D) Median frequencies of circulating follicular helper CD4+ T (Tfh) cells (CD3+CD4+CXCR5+) among total CD4+ T cells from females (rhombus) and males (stars). (E) Frequencies of intracellular ki67 expression in circulating Tfh cells from females (light) and males (dark) shown by box and whisker plots (median with interquartile range). (F) Correlation analysis between Tfh cell cycling expression on day 0 (D0) and avidity index of influenza-specific antibody on D0 in HIV-infected subjects. (G) Correlation analysis between Tfh cell cycling expression on D7

and avidity index of influenza-specific antibody on day 7 (D7) in HIV-infected subjects. r = Spearman correlation coefficient.

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Figure 4. Plasma microbial translocation and microbiome are related to influenza-specific antibody avidity in HIV-infected subjects

(A) Plasma lipopolysaccharide (LPS) levels in females (light) and males (dark) shown by box and whisker plots (median with interquartile range). (B) Correlation analysis between LPS levels on day 0 (D0) and avidity index of influenza-specific antibody at D0 in HIV-infected subjects. (C) Correlation analysis between LPS levels on D0 and avidity index of influenza-specific antibody at day 14 (D14) in HIV-infected subjects. (D) *MyD88* mRNA gene relative expression in females (light) and males (dark) shown by box and whisker plots

(median with interquartile range). **(E)** Correlation analysis between *MyD88* gene relative expression and avidity index of influenza-specific antibody at D0 in HIV-infected subjects. **(F)** Simpson diversity index comparing bacterial communities in plasma from different sexes in HIV-infected subjects and healthy controls. **(G)** Average relative abundance of each type of bacteria at the phylum level. **(H)** Relationships between bacteria at the species level and avidity of influenza-specific antibody in HIV-infected subjects. In each line, the OTUs name were shown from phylum level to species level. Inverse correlations are shown in gray highlighting; direct correlations are shown without highlighting.

Table 1

Clinical Characteristics of Study Subjects.

Medians (interquartile range)	HIV-infected Subjects (N = 26)			Healthy Controls (N = 16)		
	Male (N=17)	Female (N=9)	P	Male (N=5)	Female (N=11)	P
Age, years	36 (27–50)	47 (43–55)	0.08	32 (28–43)	49 (37–57)	0.047
Current CD4+ T-cell counts, cells/ μ L	594 (433–721)	488 (321–704)	0.52	401 (318–722)	874 (554–1,076)	0.09
Previous influenza vaccines since 2007	7 (3–9)	7 (5–8)	0.68	N/A*	N/A	N/A
Diagnosis of HIV infection, years	10 (6–10)	10 (10–18)	0.05	N/A	N/A	N/A
Duration of ART, years [†]	4 (3–6)	6 (4.5–6)	0.17	N/A	N/A	N/A
Nadir CD4+ T-cell count, cells/ μ L	361 (232–470)	204 (108–425)	0.28	N/A	N/A	N/A

* Not available/applicable. ART, antiretroviral therapy

[†] Information from our local clinic database