

# Reevaluation of immune activation in the era of cART and an aging HIV-infected population

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Biological aging is associated with immune activation (IA) and declining immunity due to systemic inflammation. It is widely accepted that HIV infection causes persistent IA and premature immune senescence despite effective antiretroviral therapy and virologic suppression; however, the effects of combined HIV infection and aging are not well defined. Here, we assessed the relationship between markers of IA and inflammation during biological aging in HIV-infected and -uninfected populations. Antibody response to seasonal influenza vaccination was implemented as a measure of immune competence and relationships between IA, inflammation, and antibody responses were explored using statistical modeling appropriate for integrating high-dimensional data sets. Our results show that markers of IA, such as coexpression of HLA antigen D related (HLA-DR) and CD38 on CD4<sup>+</sup> T cells, exhibit strong associations with HIV infection but not with biological age. Certain variables that showed a strong relationship with aging, such as declining naive and CD38<sup>+</sup> CD4 and CD8<sup>+</sup> T cells, did so regardless of HIV infection. Interestingly, the variable of biological age was not identified in a predictive model as significantly impacting vaccine responses in either group, while distinct IA and inflammatory variables were closely associated with vaccine response in HIV-infected and -uninfected populations. These findings shed light on the most relevant and persistent immune defects during virologic suppression with antiretroviral therapy.

## Introduction

Due to advances in combination antiretroviral therapy (cART), many HIV-infected individuals, especially those living in resource-rich settings, are surviving to old age. Despite virologic control of HIV, individuals on cART are susceptible to aging-associated diseases such as cardiovascular disease, kidney disease, neurocognitive decline, and cancer at earlier ages (1), suggesting premature aging in this population. Epigenetic modeling studies on blood cells have shown that cART-treated HIV-infected individuals display an approximately 5-year age acceleration compared with healthy controls (2, 3). Correlates of non-AIDS morbidity and mortality in cART-treated HIV<sup>+</sup> individuals include markers of innate and adaptive immune activation (IA) and inflammation, which can persist despite cART-induced recovery of CD4<sup>+</sup> T cell counts (4–6). Innate biomarkers of non-AIDS morbidity and mortality include serum proteins such as interleukin-6 (IL-6), C-reactive protein (CRP), and D-dimer (7, 8). Microbial translocation contributes to persistent IA in chronic HIV infection, and the biomarkers associated with it such as lipopolysaccharide (LPS), bacterial DNA, and markers of monocyte activation (e.g., soluble CD14) strongly associate with mortality and do not completely normalize under cART (9, 10). Adaptive markers of chronic IA are often limited to coexpression of HLA antigen D related (HLA-DR) and CD38 on the surface of T lymphocytes whose relative expression levels, especially on CD8<sup>+</sup> T cells, correlate with disease progression in untreated HIV infection, microbial translocation, and inflammation (10–13). As with all measurements of IA, cART initiation leads to a decrease in CD38<sup>+</sup>HLA-DR<sup>+</sup> T cells, although they are maintained at higher frequencies compared with HIV-uninfected individuals (4, 6, 14).

Biological aging is also associated with declining immunity and thus shares certain characteristics with chronic HIV infection. Aging-associated immune dysfunction in the general population is often termed immune senescence and refers to a collection of immune defects affecting multiple cell types that have been observed mostly in cohorts of very old individuals (>80 years). With regard to T cells, immune senescence is

**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Submitted:** June 21, 2017

**Accepted:** September 20, 2017

**Published:** October 19, 2017

**Reference information:**

*JCI Insight.* 2017;2(20):e95726.

<https://doi.org/10.1172/jci.insight.95726>.

insight.95726.

**Table 1. Study participant characteristics**

|                               | HIV Negative |           |           | HIV Positive |           |           |
|-------------------------------|--------------|-----------|-----------|--------------|-----------|-----------|
|                               | Young        | Middle    | Old       | Young        | Middle    | Old       |
| <b>Number of Individuals</b>  | <b>42</b>    | <b>57</b> | <b>42</b> | <b>28</b>    | <b>67</b> | <b>45</b> |
| <b>Female, n (%)</b>          | 50           | 46        | 40        | 39           | 45        | 31        |
| <b>Age, years</b>             |              |           |           |              |           |           |
| <b>Mean</b>                   | 30.1         | 51.4      | 65.1      | 29.3         | 51.5      | 64.9      |
| <b>Range</b>                  | 19–39        | 41–59     | 60–77     | 19–39        | 41–59     | 60–77     |
| <b>Duration of ART, years</b> |              |           |           |              |           |           |
| <b>Available data, n</b>      | N/A          | N/A       | N/A       | 17           | 45        | 35        |
| <b>Mean</b>                   |              |           |           | 5.94         | 11.11     | 12.69     |
| <b>Range</b>                  |              |           |           | 2–14         | 2–29      | 2–27      |
| <b>Ethnicity, n (%)</b>       |              |           |           |              |           |           |
| <b>Hispanic</b>               | 38           | 30        | 31        | 43           | 24        | 27        |
| <b>Non-Hispanic</b>           | 57           | 65        | 60        | 57           | 76        | 64        |
| <b>Not Specified</b>          | 5            | 5         | 9         | 0            | 0         | 9         |
| <b>Race, n (%)</b>            |              |           |           |              |           |           |
| <b>White</b>                  | 48           | 42        | 55        | 32           | 27        | 31        |
| <b>Black</b>                  | 38           | 54        | 33        | 61           | 72        | 67        |
| <b>Asian</b>                  | 12           | 2         | 12        | 0            | 0         | 0         |
| <b>Not Specified</b>          | 2            | 2         | 0         | 7            | 1         | 2         |

and vaccine-induced Ab production in postmenopausal women (22). The findings presented herein demonstrate that a core IA and inflammatory signature of aging is unaltered during treated HIV infection, and additionally that HIV infection drives IA independently of biological age even in the context of viral suppression with cART. Using modeling, we further show that in HIV-infected individuals, IA profiles in T cells can predict low Ab responses to influenza vaccination.

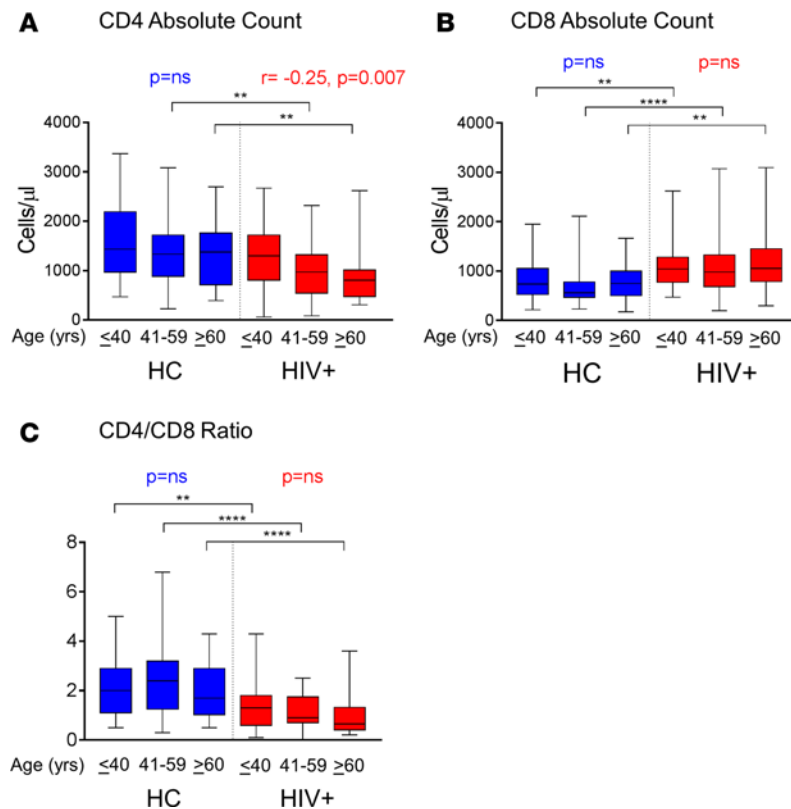
## Results

*CD4<sup>+</sup>/CD8<sup>+</sup> ratios are reduced in all age groups in HIV<sup>+</sup> compared with age-matched controls.* Prevacination data were collected from study participants in order to obtain a cross-sectional analysis of immune status in HIV-infected (HIV<sup>+</sup>) and HIV-uninfected (healthy control, HC) adults divided into 3 age groups: young, 40 years or younger; middle, 41–59 years; and old, 60 years or older (Table 1). Age, gender, and ethnicity distributions were similar in HIV-infected and -uninfected groups, although race distribution was significantly different with fewer whites in the HIV<sup>+</sup> group ( $P < 0.05$  by Student's *t* test). The CD4<sup>+</sup>/CD8<sup>+</sup> ratio is a marker of immune dysfunction in the general population as well as in HIV-infected individuals (23); therefore, we first evaluated CD4<sup>+</sup> and CD8<sup>+</sup> T cell counts and calculated the CD4<sup>+</sup>/CD8<sup>+</sup> ratio. Absolute CD4<sup>+</sup> T cell counts were significantly reduced in HIV<sup>+</sup> in the middle and old age groups and CD4<sup>+</sup> count showed an inverse correlation with biological age in the HIV<sup>+</sup> group only (Figure 1A). Meanwhile, CD8<sup>+</sup> T cell counts were significantly elevated in HIV<sup>+</sup> compared with HC in all age groups (Figure 1B). The CD4<sup>+</sup>/CD8<sup>+</sup> ratio was significantly reduced in all age groups for HIV<sup>+</sup> compared with HC but did not show a correlation with biological age in either HIV<sup>+</sup> or HC (Figure 1C).

*CD4<sup>+</sup> T cell compartment subset distribution changes with aging.* We evaluated the effects of aging and HIV infection on frequencies and distribution of CD4<sup>+</sup> T cell maturation subsets (naive, N; central memory, TCM; transitional memory, TTM; effector memory, TEM; and effector, TEff) by multiparameter flow cytometry in the participant groups (Figure 2A). In HC and HIV<sup>+</sup>, the data showed inverse correlations between naive cell frequencies and age (Figure 2B), while positive correlations were observed between memory and terminally differentiated subsets (TEM and TEff) and age (Figure 2, D and E). TTM positively

characterized by a reduced ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cells, reduced naive cells, and accumulation of effector and memory cell subsets with narrow TCR repertoires (15–17). Additionally, proinflammatory soluble protein and cytokine profiles in serum such as increased CRP and IL-6 correlate with incidence of age-associated diseases, and characterize the term inflamm-aging (18, 19). The overall result of age-related immune dysfunction from immune senescence and inflamm-aging is vulnerability to new and recrudescing infectious disease and diseases associated with aging (20, 21).

Given the overlap in biomarkers related to immune dysfunction in HIV infection and aging in the general population, the first objective of the current study was to evaluate the independent and combined effects of each state (i.e., aging and HIV) on measurements of IA and inflammation. The second objective was to determine how these measures affect immune responses to in vivo stimuli, such as the seasonal influenza vaccine, to follow up on a previous study showing that chronic HIV infection exacerbated age-related defects in B cell expansion



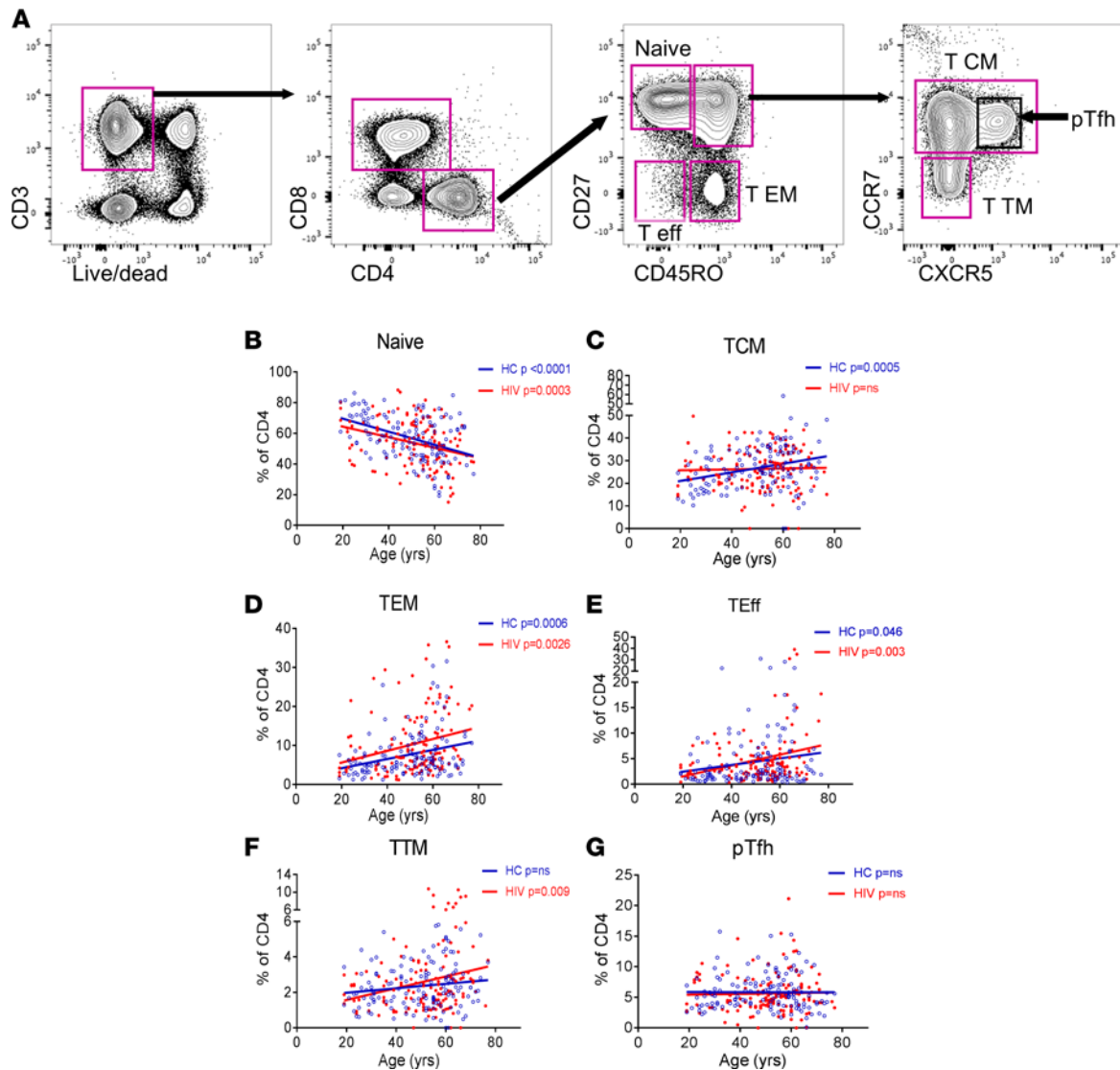
**Figure 1. Absolute CD4<sup>+</sup> and CD8<sup>+</sup> T cell counts by age group.** (A) CD4<sup>+</sup> T cell counts, (B) CD8<sup>+</sup> T cell counts, and (C) CD4<sup>+</sup>/CD8<sup>+</sup> ratio were calculated per  $\mu$ l of whole blood in each age group for HIV-negative (HC) and HIV<sup>+</sup> groups. Data are shown as box-and-whisker plots and 2-tailed Student's *t* test was used to measure statistical differences between HIV<sup>+</sup> and HC. \*\**P* < 0.01, \*\*\*\**P* < 0.0001. Pearson correlation analysis was performed between each measure and biological age (years) and results are designated by blue- or red-font *P* values for HC and HIV<sup>+</sup> groups, respectively. ns, not significant.

correlated with age in HIV<sup>+</sup> and TCM did so in HC (Figure 2, C and F). The association of TCM and TTM with age was inverted between HC and HIV<sup>+</sup>, marked by increased TCM frequencies in HIV<sup>+</sup> young. Peripheral T follicular helper (pTfh) cell frequencies (CXCR5<sup>+</sup> TCM) showed no association with age in HC or HIV<sup>+</sup> groups (Figure 2G).

*Markers of IA are elevated on CD4<sup>+</sup> T cells during controlled HIV infection.* To determine the relationships between IA and aging we analyzed the following markers of IA by multiparameter flow cytometry in total CD4<sup>+</sup> and CD8<sup>+</sup> T cells: CD38, HLA-DR, PD-1, ICOS, and Ki-67. CD38 and HLA-DR coexpression marks activated T cells and is associated with HIV disease progression. PD-1 is an immune checkpoint molecule involved in downregulating immune responses; its

expression is induced upon activation in CD4<sup>+</sup> and CD8<sup>+</sup> T cells (24). ICOS signaling is important for humoral immune responses and has been shown to be upregulated on activated CD4<sup>+</sup> T cells from HIV-infected patients (25, 26). Ki-67 is a nuclear marker of proliferation and activation that has been shown to correlate with CD4<sup>+</sup> T cell decline during HIV infection (27). We determined whether individual IA marker expression correlates with age in HC and HIV<sup>+</sup> groups and evaluated whether HIV status and aging show an interaction with regards to IA marker expression (Table 2). These analyses showed a strong reduction of CD38 expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells with age and this relationship was independent of HIV infection (Table 2 and Figure 3A). Interestingly, we observed a significant interaction between HIV infection with HLA-DR expression on CD4<sup>+</sup> T cells and age (i.e., increased with age in HIV vs. no change with age in HC, Table 2 and Figure 3B). PD-1 and ICOS expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells demonstrated moderate positive correlations with age in HC that were not observed in HIV<sup>+</sup>, due to higher expression in HIV<sup>+</sup> young individuals compared with young HC (Table 2, and Figure 3, C and D). There was also a statistical interaction between HIV infection and the change in ICOS expression with age in CD8<sup>+</sup> T cells (*P* = 0.0049) and a similar trend was observed in CD4<sup>+</sup> T cells (*P* = 0.06). Comparison of individual IA marker expression on CD4<sup>+</sup> T cells in each group showed that HIV<sup>+</sup> expressed higher levels of IA markers compared with HC, although the age at which the differences reached significance varied by marker (Figure 3).

Coexpression of CD38 and HLA-DR on CD4<sup>+</sup> and CD8<sup>+</sup> T cells was not associated with age in either participant group (Figure 4A). However, HIV<sup>+</sup> exhibited significantly higher frequencies of CD38<sup>+</sup>HLA-DR<sup>+</sup> CD4<sup>+</sup> T cells compared with HC, while the frequencies of CD38<sup>+</sup>HLA-DR<sup>+</sup> CD8<sup>+</sup> T cells were similar between HIV<sup>+</sup> and HC. To further investigate IA marker coexpression on CD4<sup>+</sup> T cells we performed Boolean combination analysis to assess the frequency of CD4<sup>+</sup> T cells expressing 5, 4, 3, 2, 1, or none of the 5 markers analyzed (CD38, HLA-DR, PD-1, ICOS, and Ki-67) (Figure 4B). CD38-single-positive cells were removed from the analysis because of their strong relationship with age in both the HIV<sup>+</sup> and HC groups (Table 2). In agreement with results showing individual expression of IA markers, in each age group HIV<sup>+</sup> individuals had higher frequencies of CD4<sup>+</sup> T cells coexpressing 2 or more IA markers compared with HC and consequently, fewer cells expressing none of the IA markers. Significant differences were not observed when combination analysis was performed in CD8<sup>+</sup> T cells (data not shown). Taken together, these data confirm and extend prior observations that cART-mediated virologic suppression does not fully reverse IA in CD4<sup>+</sup> T cells in any age group.



**Figure 2. CD4<sup>+</sup> T cell compartment subset distribution during aging and HIV infection.** (A) Gating scheme for analysis of CD4<sup>+</sup> T cell subsets by multiparameter flow cytometry. (B–G) x-y plots are shown plotting biological age (years) and individual CD4<sup>+</sup> T cell subset frequencies for each study participant: (B) naive, (C) central memory (TCM), (D) effector memory (TEM), (E) effector (TEff), (F) transitional memory (TTM), and (G) peripheral T follicular helper (pTfh) cells. P values displayed indicate results from linear regression analysis for HIV-negative (blue, HC) and HIV<sup>+</sup> (red, HIV) study participants. ns, not significant.

*Plasma biomarkers in HIV and aging.* In order to address the relationship between T cell IA and markers of inflammation, we quantified levels of soluble proteins in plasma from all study participants and evaluated their relationship to age and HIV infection status (Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.95726DS1>). First, we determined which markers were associated with biological age (Table 3). Six proteins showed significant increases with age in both the HC and HIV<sup>+</sup> groups (neopterin, sVCAM, sCD14, sCD163, soluble TNF receptor 1 [sTNFR1], and sTNFR2). Additional biomarkers were associated with age in HC only (sCD25, MCP1, sICAM, and IL-17A), while IL-8 was uniquely shown to increase with age in HIV<sup>+</sup> individuals. Next, we compared plasma levels in HIV<sup>+</sup> relative to HC individuals by age group (Figure 5). The most significant differences were observed between HIV<sup>+</sup> and HC young groups rather than middle and old, and only sTNFR2 was significantly elevated in HIV<sup>+</sup> individuals at all ages. In contrast, cytokines IL-6 and IL-21, and the cardiovascular-related biomarker brain natriuretic peptide (BNP) were lower in HIV<sup>+</sup> compared with HC at all ages. These data suggest that the plasma milieu is perturbed by HIV infection and affects the young age group more dramatically than the old when compared with HIV-uninfected individuals.

**Table 2. Correlation of IA marker expression and biological age**

|                                | HIV Negative         |                   | HIV positive         |                   | Interaction<br>P value |
|--------------------------------|----------------------|-------------------|----------------------|-------------------|------------------------|
|                                | Correlation with Age |                   | Correlation with Age |                   |                        |
|                                | r value              | P value           | r value              | P value           |                        |
| <b>CD4<sup>+</sup> T cells</b> |                      |                   |                      |                   |                        |
| CD38 (%)                       | <b>-0.41</b>         | <b>&lt;0.0001</b> | <b>-0.42</b>         | <b>&lt;0.0001</b> | 0.83                   |
| HLA-DR (%)                     |                      | 0.45              | <b>0.36</b>          | <b>&lt;0.0001</b> | <b>0.0012</b>          |
| PD-1 (%)                       | <b>0.21</b>          | <b>0.0144</b>     |                      | 0.10              | 0.63                   |
| ICOS (%)                       | <b>0.31</b>          | <b>0.0003</b>     |                      | 0.60              | 0.06                   |
| Ki-67 (%)                      |                      | 0.62              |                      | 0.17              | 0.16                   |
| <b>CD8<sup>+</sup> T cells</b> |                      |                   |                      |                   |                        |
| CD38 (%)                       | <b>-0.41</b>         | <b>&lt;0.0001</b> | <b>-0.34</b>         | <b>&lt;0.0001</b> | 0.87                   |
| HLA-DR (%)                     | <b>0.17</b>          | <b>0.05</b>       | <b>0.30</b>          | <b>0.0004</b>     | 0.23                   |
| PD-1 (%)                       | <b>0.28</b>          | <b>0.0010</b>     |                      | 0.40              | 0.18                   |
| ICOS (%)                       | <b>0.31</b>          | <b>0.0003</b>     |                      | 0.16              | <b>0.0049</b>          |
| Ki-67 (%)                      |                      | 0.10              |                      | 0.26              | 0.82                   |

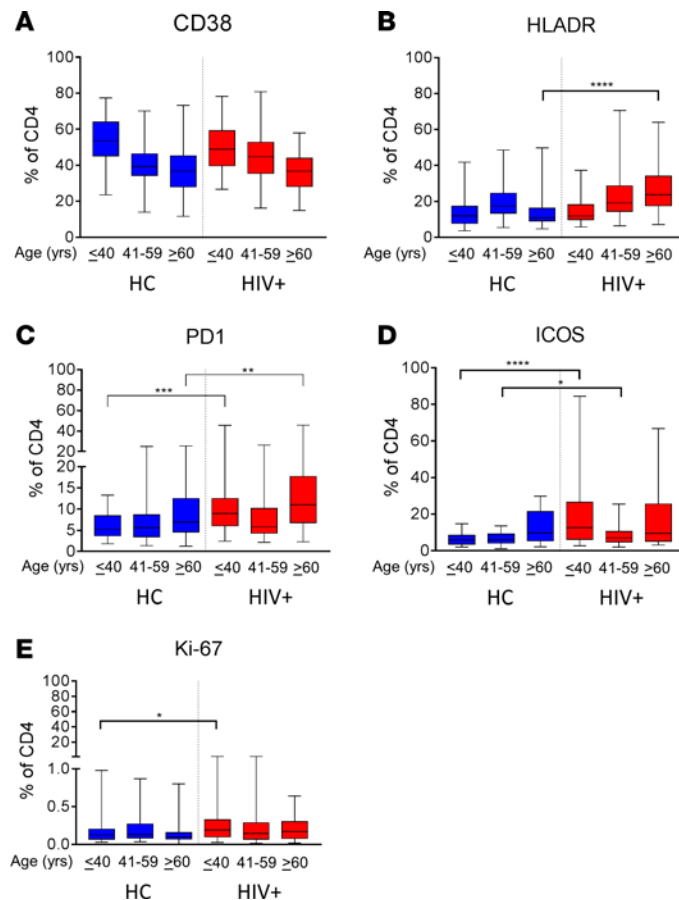
Pairwise correlation analyses were performed using all variables with significant age associations to evaluate how markers of IA and inflammation relate to one another within HC and HIV<sup>+</sup> groups. HC exhibited more age-associated variables compared with HIV<sup>+</sup> (22 vs. 17) but the overall clustering pattern was conserved (Supplemental Figure 2). Naive T cell frequencies and CD38 expression on CD4<sup>+</sup> T cells showed strong positive correlation with each other since both variables were reduced in frequency with age, and inverse correlations with all other variables. Although variables from within the same data set showed greater clustering, with flow cytometry data clustering separately from soluble biomarker data, there was a positive albeit weak correlation between cell-associated markers of IA and soluble biomarkers of inflammation in both HC and HIV<sup>+</sup>.

*HIV<sup>+</sup> individuals exhibit lower TIV response rate and magnitude compared with HC.* Based on serological Ab responses to each antigen in seasonal influenza trivalent inactivated vaccine (TIV), wherein a 4-fold increase in titer is considered a positive response, we classified participants based on response to all vaccine antigens as absolute responders or nonresponders, and those with response to only 1 or 2 antigens as other. Mean titers for participants grouped by age and responder status for each antigen before (T0) and after vaccination (T2) are presented in Supplemental Table 1. The distribution analysis showed a decline in absolute responders in

**Table 3. Correlation of plasma biomarkers with age in study participants**

|           | HIV Negative         |                               | HIV Positive         |               |
|-----------|----------------------|-------------------------------|----------------------|---------------|
|           | Correlation with Age |                               | Correlation with Age |               |
|           | r value              | P value                       | r value              | P value       |
| sCD25     | <b>0.26</b>          | <b>0.0009</b>                 |                      | 0.44          |
| MCP1      | <b>0.25</b>          | <b>0.001</b>                  |                      | 0.15          |
| sICAM     | <b>0.15</b>          | <b>0.048</b>                  |                      | 0.22          |
| IL-17A    | <b>-0.26</b>         | <b>0.0007</b>                 |                      | 0.65          |
| IL-8      |                      | 0.95                          | <b>0.20</b>          | <b>0.013</b>  |
| Neopterin | <b>0.31</b>          | <b>6.86 × 10<sup>-5</sup></b> | <b>0.22</b>          | <b>0.005</b>  |
| sVCAM     | <b>0.28</b>          | <b>0.0003</b>                 | <b>0.18</b>          | <b>0.028</b>  |
| sTNFR1    | <b>0.20</b>          | <b>0.012</b>                  | <b>0.26</b>          | <b>0.0009</b> |
| sTNFR2    | <b>0.20</b>          | <b>0.01</b>                   | <b>0.21</b>          | <b>0.011</b>  |
| sCD14     | <b>0.18</b>          | <b>0.021</b>                  | <b>0.18</b>          | <b>0.029</b>  |
| sCD163    | <b>0.19</b>          | <b>0.018</b>                  | <b>0.18</b>          | <b>0.024</b>  |

IFN- $\gamma$ , IL-6, TNF- $\alpha$ , D-dimer, LPS, BNP, CRP, and IL-21 did not show a relationship with age in HC or HIV.



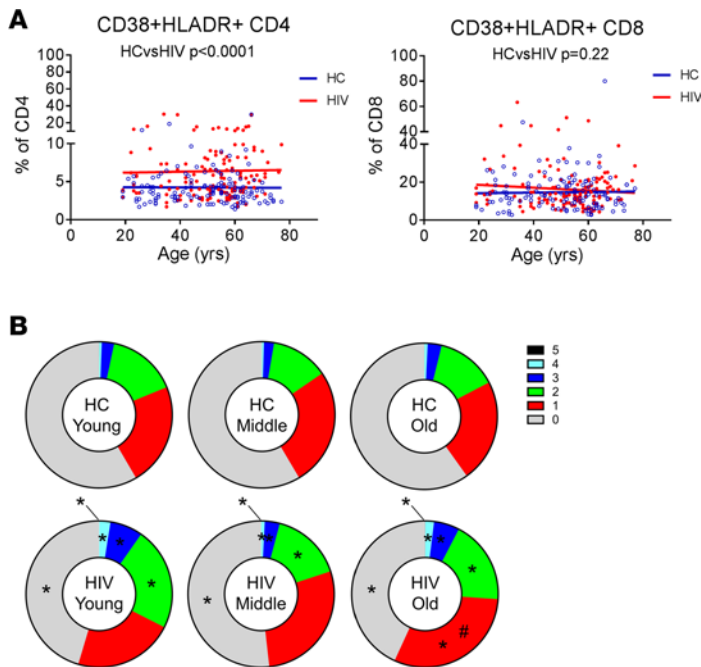
**Figure 3. Immune activation markers show higher expression in HIV<sup>+</sup> groups.** Surface expression of (A) CD38, (B) HLA-DR, (C) PD-1, (D) ICOS, and (E) Ki-67 was measured using multiparameter flow cytometry and expressed as percentage positive in total CD4<sup>+</sup> T cells by HIV status and age group. Data are shown as box-and-whisker plots and 2-tailed Student's *t* test was used to measure statistical differences between HIV<sup>+</sup> and HIV-negative (HC) for each age group. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001. Pearson correlation analysis of each marker with age within HIV<sup>+</sup> and HC are shown in Table 2.

HC with age, while in HIV<sup>+</sup> this pattern was not observed (Figure 6A). Compared with HC, there were fewer absolute responders in HIV<sup>+</sup> young and middle age groups but in the old age group they were similar. These data suggest the age-dependent decline in vaccine responses observed in HC and well-documented in the literature (28–30) is perturbed in the setting of chronic HIV infection. We assigned a response score to each participant using the combined fold increase in serum titer for all 3 antigens in order to obtain a scale on which to compare and contrast the participants. Distribution of the score across HC and HIV<sup>+</sup> participants revealed that nonresponders had response scores of 3 or lower, while absolute responders had response scores of 4 or higher (Figure 6B). HIV<sup>+</sup> participants differed from HC in that they had more individuals in the 2–3 score group and zero individuals in the 7–8 score group, highlighting the enrichment of HIV<sup>+</sup> individuals with lower Ab responses to seasonal TIV. Participants who responded to only 1 or 2 strains with a 4-fold increase in Ab titer from T0 to T2 (designated “other”) were present throughout the score scale, although these individuals were concentrated in the 3–4 score group in HC and HIV<sup>+</sup>, creating a buffer between absolute responders and nonresponders.

**LASSO modeling to determine variables associated with TIV response.** We used the statistical method LASSO (least absolute shrinkage and selection operator) (31) to generate predictive models and identify which variables in our data set closely associated with response score in HC and HIV<sup>+</sup> groups separately. LASSO was selected because it penalizes variables that highly correlate with one another to avoid overfitting the model, which was necessary considering the high degree of correlation among the variables measured including biological age (Supplemental Figure 2). Nineteen plasma biomarker parameters and 111 flow cytometry parameters were entered as *x* variables for LASSO analysis using the response score as the outcome. These analyses generated models containing unique variables for HC and HIV<sup>+</sup> groups that were able to segregate absolute responders and nonresponders (Figure 7, A and B). For HC, 4 variables were selected by the model including frequencies of pTfh, HLA-DR<sup>+</sup> CD8<sup>+</sup> T cells, and HLA-DR<sup>+</sup> ICOS<sup>+</sup> CD4<sup>+</sup> T cells, all of which had a positive association with vaccine response score (Table 4). CD8<sup>+</sup> T cell count had a negative association with Ab responses. In HIV<sup>+</sup>, 8 variables were selected including plasma levels of IL-21 and sCD25, both of which had a negative association with vaccine response (Table 4). Interestingly, 4 pTfh subsets (defined using Boolean combination analysis of IA marker expression) showed significant associations with vaccine response. The subsets that had negative associations with vaccine response had the phenotype CD38<sup>+</sup>HLA-DR<sup>+/−</sup>ICOS<sup>+/−</sup>Ki-67<sup>+</sup>PD-1<sup>+</sup>, while subsets with a positive association had the phenotype CD38<sup>+/−</sup>HLA-DR<sup>+/−</sup>ICOS<sup>+</sup>Ki-67<sup>+</sup>PD-1<sup>−</sup>. Frequencies of CD4<sup>+</sup> TCM cells and CD38<sup>−</sup>HLA-DR<sup>−</sup> CD8<sup>+</sup> T cells both also showed positive associations with vaccine response in HIV<sup>+</sup> individuals.

## Discussion

The terms IA and inflammation are often used interchangeably when referring to the immunological side-effects of HIV infection and aging. In this study we sought to shed light on the relationships between IA and inflammation in the context of HIV infection and aging, in order to address challenges associated with a burgeoning population of elderly HIV-infected individuals. We evaluated a broad range of established markers of IA and inflammation and compared and contrasted expression levels within different age groups

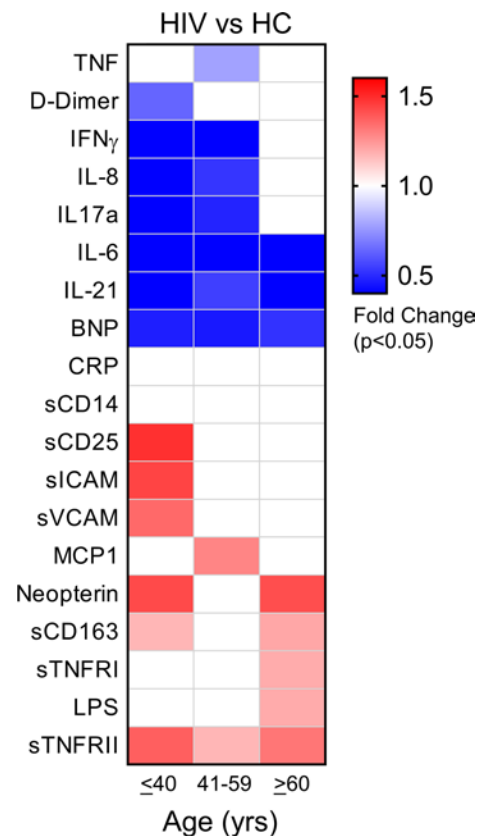


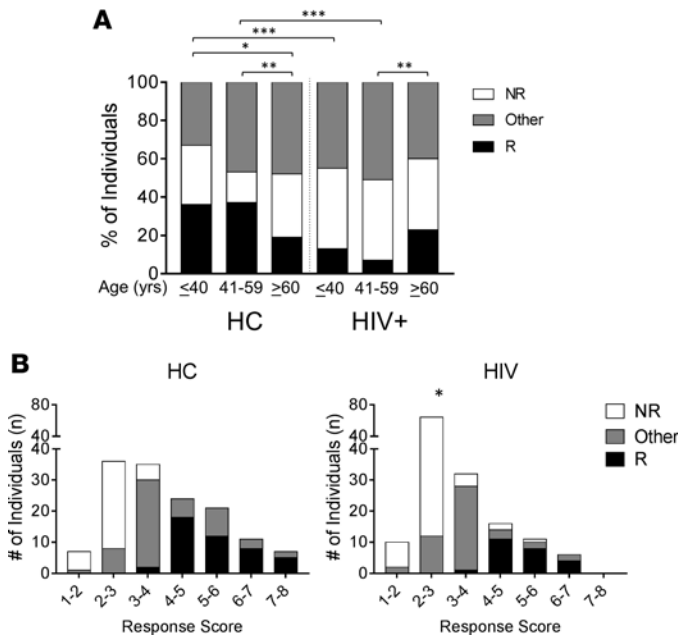
**Figure 4. Coexpression of immune activation markers on CD4<sup>+</sup> T cells at higher frequencies in HIV<sup>+</sup> individuals compared with HIV negative (HC) regardless of age.** (A) The relationship between frequencies of CD38<sup>+</sup>HLA-DR<sup>+</sup> CD4<sup>+</sup> T cells (left) and CD8<sup>+</sup> T cells (right) with age (years) is shown. *P* values indicate results of 2-tailed Student's *t* test to measure statistical differences between HIV<sup>+</sup> and HC. (B) Ring graphs represent combination gate (Boolean) analysis using the 5 immune activation markers (CD38, HLA-DR, PD-1, ICOS, Ki-67) on CD4<sup>+</sup> T cells. Each ring shows relative frequency of cells expressing 0, 1, 2, 3, 4, or 5 markers simultaneously for each group. Multiple *t* tests were performed to compare relative frequencies from one ring (i.e., study group) to another. \*Indicates significant FDR < 1% when compared with HIV<sup>+</sup> group. #Indicates significant FDR < 1% when compared with HIV young group.

in HIV-infected and -uninfected individuals. Our data revealed a core signature of aging that was unaltered by the presence of chronic HIV infection and included changes in the T cell compartment subset distribution with age and a loss of CD38 expression on T cells. However, some age-related changes in marker expression were observed only in HIV-negative individuals, including increasing cell-surface expression of PD-1 and ICOS on CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as increasing plasma biomarkers sCD25, sICAM, and MCP1. Further analysis showed that expression levels of these particular markers were already increased in younger HIV<sup>+</sup> individuals, thereby negating the effect of aging that was observed in HC. These results provide the basis for an immune signature of premature aging in HIV<sup>+</sup>, even when virological suppression is achieved.

The finding of reduced CD38 expression with age was not unexpected since it is expressed on double-positive thymocytes and thymus function declines steadily through at least age 60 (32). However, CD38 expression on T cells (especially CD8) has also been extensively studied and implicated as a correlate of HIV disease progression (33–35) and further, coexpression with HLA-DR on the surface of T cells is a widely accepted measure of IA (9, 10, 36). Therefore, we expected that HIV<sup>+</sup> would exhibit differential expression patterns of CD38 on T cells compared with HC. The data, however, showed a direct overlap of linear slopes relating CD38 expression and age in HIV<sup>+</sup> and HC groups on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Despite the remarkable overlap, both groups exhibited a broad distribution of CD38 expression within age groups, suggesting that age alone is not driving CD38 expression on T cells. Furthermore, the reduction in CD38 expression with age seemed to preferentially affect the CD38<sup>+</sup>HLA-DR<sup>-</sup> population rather than double positive (CD38<sup>+</sup>HLA-DR<sup>+</sup>), which was not associated with age in CD4<sup>+</sup> or CD8<sup>+</sup> T cells from either group. Interestingly, the double-positive (CD38<sup>+</sup>HLA-DR<sup>+</sup>) T cells were significantly higher in HIV<sup>+</sup> compared with HC in the CD4<sup>+</sup> compartment only. This finding was consis-

**Figure 5. The effect of HIV on prevaccination plasma biomarkers in study participants by age group.** Heatmap showing fold-change difference between HIV<sup>+</sup> participants and HIV negative (HC) for different age groups. Colored boxes represent significant (*P* < 0.05) differences between HIV<sup>+</sup> and HC using 2-tailed Student's *t* test. Blue color represents lower concentration in HIV<sup>+</sup> relative to HC, and red color represents higher concentration of the indicated soluble protein in HIV<sup>+</sup> relative to HC.





**Figure 6. HIV<sup>+</sup> individuals have lower response rate and magnitude against trivalent inactivated influenza vaccine compared with HIV negative (HC).** (A) Bar graph showing the distribution of absolute responders and nonresponders by age group in HIV<sup>+</sup> and HC study participants.  $\chi^2$  analysis was used to determine differences in responder status between the groups. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.01$ . (B) Distribution of response scores in HC (left) and HIV<sup>+</sup> (right) groups.  $\chi^2$  analysis was performed to compare score distributions between HC and HIV<sup>+</sup>. \* $P < 0.05$ .

tent for other IA markers tested, which exhibited increased frequency in CD4<sup>+</sup> T cells and not CD8<sup>+</sup> T cells (except HLA-DR) in HIV<sup>+</sup> compared with HC. These data demonstrate the dominant effect of HIV on IA over biological age and show that while virologic control with cART is able to normalize IA in CD8<sup>+</sup> T cells, IA persists in CD4<sup>+</sup> T cells in HIV<sup>+</sup> virologically suppressed patients. We also found that levels of IA on CD4<sup>+</sup> T cells were not associated with duration of ART treatment (data not shown), suggesting that persistent IA on CD4<sup>+</sup> T cells may be irreversible and the time of treatment initiation relative to infection (e.g., during acute infection) may be key in determining IA, as has been suggested previously (5).

An important question we wanted to address with the current study was whether any of the variables associated with biological age, IA, and inflammation, were predictive of influenza vaccine Ab responses. In a previous study of postmenopausal women with and without HIV, significantly reduced levels of postvaccination immunological measures (i.e., memory B cell and pTfh expansion, pTfh IL-21<sup>+</sup> frequencies) were observed in HIV<sup>+</sup> compared with HIV negative, leading to the conclusion that HIV exacerbated age-related immune defects, even though frequencies of responders to H1N1 were not different between the 2 groups (22). Additionally, prevaccination CD4 IA showed a positive correlation with age and inverse correlation with H1N1 response, which formed the basis of our questions regarding baseline IA and vaccine response for the current study.

The influenza strains included in the tri- or quadrivalent influenza vaccine are determined yearly based upon expected circulating strains of influenza; however, year-to-year variation in the TIV used was relatively low during the study period. The H1N1 pdm09 antigen has been present in the vaccine since the H1N1 influenza A pandemic in 2009, and H3N2 and B antigens were identical for the first 2 seasons of the study. The vast majority of individuals in our study exhibited seroprotective Ab titers (>1:40) to all antigens at baseline. The prevalence of existing humoral immune responses in the human population complicates use of the influenza vaccination model for determining immunological predictors of vaccine responsiveness. Nevertheless, many groups have used and continue to use the seasonal flu vaccine to probe immune competence and explore new biomarkers in order to improve vaccine design (29, 37–43). The use herein of absolute response and response score criteria facilitates comparisons without altering the conventional definitions for determining vaccine responder status.



**Figure 7. Discrimination of influenza vaccine responders and nonresponders using LASSO-identified variables.** Partial least squares discriminant analysis (PLSDA) plots are shown for (A) HIV-negative (HC) ( $n = 57$ ) and (B) HIV<sup>+</sup> ( $n = 64$ ) absolute responders ( $\Delta$ ) and nonresponders ( $\circ$ ) based on the variables identified by LASSO in Table 4.



**Table 4. Identification of variables associated with influenza vaccine response using LASSO****Variables associated with response score in HC**

|   | Estimate | Std. Error | t value | Pr(> t ) |
|---|----------|------------|---------|----------|
| (Intercept)                             | 6.0445   | 2.8724     | 2.104   | 0.04030  |
| CD8 absolute count                      | -0.9504  | 0.2782     | -3.416  | 0.00125  |
| pTfh (% of CD4)                         | 0.6293   | 0.2603     | 2.417   | 0.01925  |
| CD38*HLA-DR*ICOS*Ki-67*PD-1* (% of CD4) | 0.9858   | 0.3962     | 2.488   | 0.01614  |
| HLA-DR (% of CD8)                       | 0.7383   | 0.2763     | 2.672   | 0.01009  |

Residual standard error: 1.252 on 51 degrees of freedom. Multiple R<sup>2</sup>: 0.5123, adjusted R<sup>2</sup>: 0.4645. F statistic: 10.71 on 5 and 51 degrees of freedom, P value: 4.494 × 10<sup>-7</sup>.

**Variables associated with response score in HIV**

|  | Estimate  | Std. Error | t value | Pr(> t ) |
|--|-----------|------------|---------|----------|
| (Intercept)                              | -0.77351  | 2.21246    | -0.350  | 0.727963 |
| IL-21                                    | -0.16089  | 0.07367    | -2.184  | 0.033509 |
| sCD25                                    | -0.35134  | 0.12512    | -2.808  | 0.007001 |
| TCM (% of CD4)                           | 0.87210   | 0.30730    | 2.838   | 0.006461 |
| CD38*HLA-DR*ICOS*Ki-67*PD-1* (% of pTfh) | -3.13382  | 1.06869    | -2.932  | 0.004991 |
| CD38*HLA-DR*ICOS*Ki-67*PD-1* (% of pTfh) | 12.24164  | 3.90059    | 3.138   | 0.002798 |
| CD38*HLA-DR*ICOS*Ki-67*PD-1* (% of pTfh) | 4.11749   | 1.59357    | 2.584   | 0.012621 |
| CD38*HLA-DR*ICOS*Ki-67*PD-1* (% of pTfh) | -90.06714 | 34.09332   | -2.642  | 0.010868 |
| CD38*HLA-DR* (% of CD8)                  | 0.92365   | 0.23533    | 3.925   | 0.000256 |

Residual standard error: 1.094 on 55 degrees of freedom. Multiple R<sup>2</sup>: 0.4789, adjusted R<sup>2</sup>: 0.4031. F statistic: 6.318 on 8 and 55 degrees of freedom, P value: 8.669 × 10<sup>-6</sup>.

The LASSO method has been used to identify key variables from large, complex data sets contributing to particular outcomes such as protective HIV vaccine Ab profiles (44) and spontaneous control of HIV disease (45). Here, we used LASSO to resolve predictors of serological responses to TIV in HIV<sup>+</sup> and HC individuals. The results showed that certain markers of IA are associated with reduced influenza vaccine responses, as we expected, although only in the HIV<sup>+</sup> group. One of these markers, soluble CD25, is a general marker of IA and is elevated in diabetes, autoimmune disease, cancer, and immunodeficiency diseases (46). We previously showed that plasma sCD25 is elevated in HIV<sup>+</sup> compared with HC postmenopausal women (47). Although sCD25 showed a positive correlation with age in HC, it was selected as a significant negative predictor of vaccine response in the HIV<sup>+</sup> group only. All CD4, CD8, and pTfh flow cytometry variables were loaded into LASSO analysis (Supplemental Table 2) and interestingly, the pTfh cell subset stood out with meaningful associations with vaccine response in both HC and HIV<sup>+</sup>. Within germinal centers in lymph nodes, Tfh cells produce IL-21 and provide a number of signals to B cells through cell-surface receptor–ligand interactions. Although pTfh frequencies were similar between HC and HIV<sup>+</sup>, IL-21 levels in plasma were significantly reduced in HIV<sup>+</sup> compared with HC, especially in the young and middle age groups, and paradoxically we found that baseline plasma IL-21 levels were negatively associated with vaccine response in the HIV<sup>+</sup> group only. In a previous study in HIV<sup>+</sup> children we found that baseline levels of *IL21R* gene expression in pTfh were negatively associated with vaccine response to H1N1 (38). The relevance of these data are unclear and may point to aberrant IL-21 signaling in HIV<sup>+</sup> individuals that may be inhibitory for immune function and especially Tfh-mediated B cell responses to vaccination. Induction of IL-21 following vaccination may be a more relevant biomarker, as IL-21–producing CD4<sup>+</sup> T cells in the circulation, recognized as pTfh, have been shown to correlate with Ab responses to vaccination (22, 48, 49). We found an association of pTfh frequency with vaccine response in HC, while in HIV<sup>+</sup>, specific IA marker expression on pTfh was predictive and cell frequency was not associated. Of the 5 IA markers evaluated using Boolean gating on pTfh cells, PD-1 expression was clearly associated with low Ab response to TIV. Coexpression of CD38 and Ki-67 was required for the observed negative association, while PD-1–negative pTfh cells coexpressing ICOS and HLA-DR showed positive associations with TIV response. Collectively, these data substantiate previous

findings that functional defects in pTfh cells present prior to vaccination in HIV-infected individuals on cART contribute to poor vaccine-induced Ab production (38, 50) and support peripheral Tfh cells as a promising biomarker for assessing immune function and potential for Ab response in response to vaccination. The PD-1/PD-L1 signaling axis between T/B cells has recently been implicated in dysregulated B cell function and Ab response to TIV by our group (28). Indeed, the finding of PD-1, an immune checkpoint molecule, as a predictive marker on pTfh provides a potential target for intervention given the therapeutic successes of checkpoint-inhibitor drugs, and strategies targeting this pathway may lead to improved immune function in HIV<sup>+</sup> individuals.

The CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio has been shown to correlate with immune function in a number of clinical settings, especially in HIV infection. The volunteer population in this study matched other cohorts exhibiting reduced CD4<sup>+</sup> T cell absolute counts and CD4<sup>+</sup>/CD8<sup>+</sup> ratios and increased CD8<sup>+</sup> T cell counts in cART-treated HIV<sup>+</sup> individuals compared with HC (51), although neither of these variables were selected in LASSO analysis for prediction of vaccine response in HIV<sup>+</sup>. In HC, however, CD8<sup>+</sup> T cell counts were selected as a negative predictor of response. A decline in CD4<sup>+</sup> T cell count with age in HIV-negative individuals has been documented previously (52); although our data did not reach significance for the correlation between CD4<sup>+</sup> T cell count and age, there was a downward trend. The data shown here suggest that CD8<sup>+</sup> T cell counts may be a more specific marker of immune dysfunction than CD4<sup>+</sup> T cell count or the ratio in HC. In line with results showing an increase in PD-1 with age on CD8<sup>+</sup> T cells in HC (Table 2), we found a moderate negative association of PD-1 expression on CD8<sup>+</sup> TEM with vaccine response score ( $r = -0.31$ ,  $P = 0.0002$ , data not shown), which could contribute to the impact of CD8<sup>+</sup> T cell count observed in Table 4. Despite the differences in T cell counts between HC and HIV<sup>+</sup>, the changes in distribution of T cell maturation subsets with age were largely conserved in the context of cART-treated HIV infection, with the exception of CD4<sup>+</sup> TCM and TTM which demonstrated differential relationships with aging in HIV<sup>+</sup> and HC. In HIV<sup>+</sup>, TTM increased with age and showed no change in TCM, while the opposite pattern was observed in HC. This subtle difference in the memory compartment may indicate a decreased potential for proliferation, self-renewal, and lymphoid homing of CD4<sup>+</sup> T cells in aging HIV<sup>+</sup> individuals (53). TCM frequency was positively associated with vaccine response in HIV<sup>+</sup>, lending further support to this hypothesis.

Plasma levels of CRP, D-dimer, and IL-6 have all been shown to be biomarkers of non-AIDS comorbidities in HIV-infected individuals, even those on cART (52). In our study population, CRP and D-dimer levels did not exhibit linear relationships with age in either HC or HIV<sup>+</sup>, although they did show increased levels in the middle and old groups compared with young (Supplemental Figure 1). Interestingly, IL-6 levels were reduced in the HIV<sup>+</sup> group compared with HC. These data suggest that biomarkers for Ab response to vaccination differ from those involved in comorbid health events such as malignancy, nonaccidental mortality, cardiovascular disease, and stroke.

Overall, our data demonstrate that HIV-related immune senescence, which is age independent and characterized by increased IA expression on CD4<sup>+</sup> T cells, is distinct from biological aging. Further, in terms of evaluating immune response to vaccinations, the same variables were not predictive in both HC and HIV<sup>+</sup> individuals, so it will be necessary to consider these populations separately in regards to vaccine strategies. In the case of HC, expansion of pTfh cells could improve vaccine response, while in HIV<sup>+</sup> it will be important to modify the quality of pTfh in order to improve the outcome. The use of animal models such as humanized mouse models and nonhuman primate models for HIV may be useful to test interventions for boosting immune function (54, 55). Future studies should aim to understand how pTfh function is affected by persistent IA and inflammation in HIV-infected individuals with a focus on antigen-specific pTfh.

## Methods

**Study volunteers.** Study participants were recruited from University of Miami, Jackson Memorial, and VA Hospitals in Miami, FL. Participants were recruited in 2 populations: (a) 140 HIV-infected, cART-treated individuals who demonstrated virus suppression (HIV RNA < 40 copies/ml) for at least 1 year prior to enrollment, and (b) 141 HIV-uninfected healthy controls. Individuals were enrolled concurrently from 3 age groups: young (18–39 years), middle age (40–59 years), and old (60 years or older). Table 1 shows sex, race, ethnicity, and duration of ART data for each applicable participant group. Peripheral blood mononuclear cells (PBMCs) and plasma were stored in liquid nitrogen and –80°C freezers, respectively, until further experiments were performed.

*Ethics statement.* The study was approved by the University of Miami Institutional Review Board. Voluntary signed informed consent was obtained from every participant prior to participating in the study.

*Influenza vaccine study.* All participants were administered the seasonal influenza TIV and provided peripheral blood samples at prevaccination (T0) and postvaccination time points: day 7 (T1), day 21 (T2), and week 24 (T3). Serum titers for Abs against each vaccine strain were determined at every time point by hemagglutination inhibition assay as described previously (56). Individual vaccine strain antigens were provided as gifts from Giuseppe del Giudice (Novartis, Siena, Italy). For the 2013–2014 and 2014–2015 seasons the 3 strains in the vaccine were H1N1 A/California/7/2009, H3N2 A/Texas/50/2012, and B/Massachusetts/02/2012-like. For the 2015–2016 season the 3 strains were H1N1 A/California/7/2009, H3N2 A/Switzerland/9715293/2013, and B/Phuket/3073/2013.

*Multiparameter flow cytometry.* Cryopreserved PBMCs were thawed in groups of 18 study participants at a time, randomized by group affiliation (HIV status and age group). Thawed PBMCs were rested overnight in RPMI including 10% fetal bovine serum, then washed and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) prior to labeling for cell surface and intracellular protein expression using commercially available fluorescent marker-conjugated mAbs. IA markers were evaluated in T cell subsets in 1 tube containing the following Abs against cell surface antigens obtained from BD Biosciences: CD3 (564001/clone SK7), CD4 (552838/clone L200), CD8 (561453/clone RPA-T8), CD45RO (561137/clone UCHL1), CCR7 (562381/clone 150503), CXCR5 (558113/clone RF8B2), CD38 (555460/clone HIT2), and PD-1 (564104/clone EH12.1). The following additional Abs were obtained from Biolegend: HLA-DR (307604/clone L243) and ICOS (313524/clone C398.4A). Anti-CD27 (15-0279-41/clone 323) was obtained from eBiosciences. Following surface labeling, cells were permeabilized and fixed using BD Cytofix/Cytoperm and labeled for intracellular expression of Ki-67 (350516/clone Ki-67 from Biolegend). Samples were fixed with 1% paraformaldehyde in PBS and stored at 4°C for up to 24 hours before acquisition on a BD Fortessa instrument. Flow cytometry data were analyzed manually using FlowJo V10 (Tree Star, Inc.).

*Plasma assays.* Plasma levels of sCD14, sCD163, sCD25, sTNFR1, sTNFR2, and CRP were determined using the Human Quantikine Immunoassay (R&D Systems) following the manufacturer's instructions. For sCD14 measurement, samples were diluted 400-fold and results are expressed in pg/ml. For sCD163 measurement, samples were diluted 30-fold and results are expressed in pg/ml. For sCD25 measurement, samples were diluted 4-fold and results are expressed in pg/ml. For sTNFR1 and sTNFR2 measurement, samples were diluted 10-fold and results are expressed in pg/ml. For CRP measurement, samples were diluted 200-fold and results are expressed as pg/ml. IL-21 plasma levels were determined on undiluted samples (Biolegend) and results are expressed in pg/ml. BNP plasma levels were determined on undiluted samples (Eagle Biosciences) and results are expressed in pmol/ml. Neopterin plasma levels were determined on undiluted samples (IBL) and results are expressed in pg/ml. For D-dimer measurement (Sekisui), plasma samples were diluted 50-fold and results are expressed in pg/ml.

*LPS measurement.* LPS levels were measured in plasma samples by the use of the Limulus amoebocyte lysate chromogenic endpoint assay (Lonza Group Ltd) according to the manufacturer's recommendations. Samples were diluted 1:5 in endotoxin-free water and heat inactivated at 80°C for 10 minutes prior to the assay. LPS concentration in the samples was calculated in relation to an *E. coli* endotoxin standard and expressed in pg/ml.

*Multiplex cytokine measurement.* Plasma levels of cytokines were measured using a customized MILLI-PLEX Human Cytokine magnetic bead panel (EMD Millipore) following the manufacturer's instructions. Briefly, plasma samples were thawed, vortexed, and centrifuged at 1,000 *g* for 3 minutes immediately prior to testing. Undiluted plasma was incubated overnight with a mixture of beads specific for IL-6, IL-8, IL-17A, IFN- $\gamma$ , MCP-1, and TNF- $\alpha$  at 4°C with shaking. Plasma s-ICAM1 and sVCAM-1 were measured in the same manner using a customized MILLI-PLEX Human Neurodegenerative Disease magnetic bead panel (EMD Millipore). After washing, the beads were incubated with biotinylated detection Abs for 1 hour at room temperature. Streptavidin-PE was then added to the wells and allowed to incubate for 30 minutes at room temperature. The beads were then washed and diluted with 150  $\mu$ l Sheath Fluid before acquisition on a MAGPIX instrument (Luminex Corporation). The mean fluorescence intensity (MFI) data were analyzed with MILLI-PLEX Analyst Software V.3.5 (EMD Millipore). Cytokine concentrations were determined based on standard curves and expressed in pg/ml.

*Determining serological response to influenza vaccine.* The serological response to each vaccine strain was defined as a 4-fold or greater increase in titer at T2 relative to T0. We used the term absolute responder to identify participants with a 4-fold or greater increase in serum titer for all 3 strains and absolute nonresponder

to identify participants with a less than 4-fold increase in titer for all 3 strains. The term “other” identifies participants who responded to 1 or 2 antigens only. A response score was determined for each participant for use in correlation analysis with flow cytometry and plasma biomarker data sets using the following formula: score =  $\log_2(\text{H1N1 fold change T2/T0} + \text{H3N2 fold change T2/T0} + \text{B fold change T2/T0})$ . The score allowed us to combine responses from all 3 strains into 1 data point while retaining information regarding magnitude of response to 1 or more strains.

**Statistics.** All data sets were loaded into Prism version 7.00 for Windows (GraphPad Software) and variables related to T cell counts, multiparameter flow cytometry data, and plasma biomarkers were evaluated for associations with biological age using Pearson correlation analyses and by age group between HC and HIV<sup>+</sup> using a 2-tailed Student's *t* test. A *P* value less than 0.05 was considered significant. Box-and-whisker plots were generated in Prism and the bounds of the box extends from the 25th to 75th percentile and the line inside the box is the median value. The whiskers represent minimum and maximum values. Pair-wise correlation matrix analysis was implemented using the R package corrgram and variables in the correlogram were ordered using the principal component analysis. For association of variables with influenza vaccine response, 131 variables listed in Supplemental Table 2 were  $\log_2$  transformed and loaded into R package glmnet for LASSO analysis to fit a generalized linear model via penalized maximum likelihood and regression regulated at  $\alpha = 1$  (57). Ordinary least squares was performed on LASSO lambdaMin-selected variables to reveal the association with response score and partial least squares discriminant analysis (PLS-DA) was performed to predict categorical response (responders, nonresponders) using R package mixOmics (58). Note: In our data set, individuals with very high baseline titers (>1:320) were always characterized as nonresponders due to the inability to reach a 4-fold increase above baseline; therefore, we implemented a cutoff of 1:320 for baseline titer for participants to be included in the LASSO modeling analysis.

**Study approval.** All subjects (Table 1) were deidentified and provided written informed consent according to protocols approved by IRB of the University of Miami in accordance with the Declaration of Helsinki.

### Author contributions

LDA, S. Pallikkuth, VG, SR, RP, and S. Pahwa provided intellectual input and contributed to the experimental design. LDA, S. Pallikkuth, VG, and SR performed the experiments. KLA and SR performed statistical analysis. LDA, S. Pallikkuth, and S. Pahwa wrote the manuscript. S. Pahwa was responsible for the overall design and conduct of the study.

### Acknowledgments

This work was supported by a National Institutes of Allergy and Infectious Diseases grant awarded to S. Pahwa (RO1AI108472) and made possible through support for the Miami Center for AIDS Research (CFAR) at the University of Miami Miller School of Medicine funded by a grant (P30AI073961) from the NIH, which is supported by the following NIH Co-Funding and Participating Institutes and Centers: NIAID, NCI, NICHD, NHLBI, NIDA, NIMH, NIA, NIDDK, NIGMS, FIC, and OAR. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

The authors would like to thank all study participants for their time and cooperation and the following clinicians for patient recruitment: Margaret Fischl, Gordon Dickinson, Allan Rodriguez, and Maria Alcaide. We thank Margaret Roach, Maria Pallin, Celeste Sanchez, Sidney Lane, and Anil Mahavadi for technical assistance in this study; and Louis Gonzalez, Katherine Klose, Valeria Boter, and the Clinical Research Center at University of Miami Hospital for assistance recruiting and managing study participants. We thank Li Pan for statistical analysis.

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