Loss of Pre-Existing Immunological Memory among HIV Infected Women
Despite Immune Reconstitution with Antiretroviral Therapy

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Despite successful immune reconstitution following antiretroviral therapy (ART), virus-specific CD4+ T cell memory and antiviral antibody responses following childhood smallpox vaccination were found to be preferentially lost among HIV+ women compared to matched HIV- controls.

**Footnote**

Conflict of Interest

The authors state that they have no financial conflicts of interest. Each of the authors had access to the data included in the study and the corresponding authors had final responsibility for the decision to submit the manuscript for publication.

**Funding**

This work was supported in part by the National Institutes of Health Public Health Service grant U19 AI109948 (MKS), and Oregon National Primate Research Center grant, 8P51 OD011092 (MKS). WIHS (Principal Investigators): Bronx WIHS (Kathryn Anastos and Anjali Sharma), U01-AI-035004; Brooklyn WIHS (Howard Minkoff and Deborah Gustafson), U01-AI-031834; Chicago WIHS (Mardge Cohen and Audrey French), U01-AI-034993; Metropolitan Washington WIHS (Seble Kassaye), U01-AI-034994; Connie Wofsy Women’s HIV Study, Northern California (Nadia Roan, Bradley Aouizerat, and Phyllis Tien), U01-AI-034989; WIHS Data Management and Analysis Center (Stephen Gange and Elizabeth Golub), U01-AI-042590; Southern California WIHS (Joel Milam), U01-HD-032632 (WIHS I – WIHS IV). The WIHS is funded primarily by the National Institute of...
Allergy and Infectious Diseases (NIAID), with additional co-funding from the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), the National Cancer Institute (NCI), the National Institute on Drug Abuse (NIDA), and the National Institute on Mental Health (NIMH). Targeted supplemental funding for specific projects is also provided by the National Institute of Dental and Craniofacial Research (NIDCR), the National Institute on Alcohol Abuse and Alcoholism (NIAAA), the National Institute on Deafness and other Communication Disorders (NIDCD), and the NIH Office of Research on Women’s Health. WIHS data collection is also supported by UL1-TR000004 (UCSF CTSA), UL1-TR000454 (Atlanta CTSA), P30-AI-050410 (UNC CFAR), and P30-AI-027767 (UAB CFAR). The funders of this study played no role in writing the manuscript or reporting/interpretation of the data.

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Abstract

Background. It is unclear if HIV infection results in permanent loss of T cell memory or if it impacts pre-existing antibodies to childhood vaccinations/infections.

Methods. We conducted a matched cohort study involving 50 pairs of HIV+ and HIV- women. Total memory T cell responses were measured after anti-CD3 stimulation or after vaccinia virus stimulation to measure T cells elicited after childhood smallpox vaccination. Vaccinia-specific antibodies were measured by ELISA.

Results. There was no difference between HIV+ and HIV- subjects in terms of CD4+ T cell responses after anti-CD3 stimulation (P=0.19) although HIV+ subjects had significantly higher CD8+ T cell responses (P=0.033). In contrast, there was a significant loss in vaccinia-specific CD4+ T cell memory among HIV+ subjects (P=0.039) whereas antiviral CD8+ T cell memory remained intact (P=1.0). Vaccinia-specific antibodies were maintained indefinitely among HIV- subjects (half-life; infinity, 95%CI, 309 years-infinity) but declined rapidly among HIV+ subjects (half-life; 39 years, 95%CI, 24-108 years, P=0.001).

Conclusions. Despite ART-associated improvement in CD4+ T cell counts (nadir CD4 <200 cells/mm³ with >350 cells/mm³ after ART), antigen-specific CD4+ T cell memory to vaccinations/infections that occurred before HIV infection did not recover after immune reconstitution and a previously unrealized decline in pre-existing antibody responses was observed.

Key words: HIV, ART, antiretroviral therapy, smallpox, vaccination, immunological memory
Background

Suppression of HIV replication using active antiretroviral therapies (ART) has allowed for restoration of immune function and many opportunistic infections, once common are now rare[1]. Despite these advancements, HIV+ individuals continue to suffer from a 4-fold greater incidence of varicella zoster[2] and other virus-associated ailments[3], leading to the possibility that immune function may still not be fully optimal. Although ART improves many aspects of immunological function[4], there is continuing debate regarding the ability of ART to restore T cell memory to vaccines or infections that were encountered prior to HIV infection. Some studies indicate that pre-existing antigen-specific T cell memory is either lost[5-7] or restored[5, 8-10] after ART-associated immunereconstitution. Furthermore, little is known about the durability of pre-existing serum antibody responses after HIV/ART[11, 12]. Since HIV+ individuals often demonstrate immunological characteristics that are more commonly associated with an aging immune system[13], this raises questions regarding whether HIV infection exacerbates immune senescence in part by decreasing protective immunological memory to vaccinations or infections that occurred in the distant past. Vaccinia virus (used during smallpox vaccination) represents an ideal antigen for determining the duration of immunity in the absence of re-infection since, a) the last case of smallpox in the U.S. occurred in 1949[14, 15], b) routine civilian smallpox vaccination was discontinued in 1972[16], and c) there are no cross-reactive orthopoxviruses in the U.S. that commonly infect humans. Moreover, vaccinia has the added advantage of inducing strong antiviral CD4+ and CD8+ T cell memory and readily infects primary monocytes[17], an antigen presenting cell (APC) found among peripheral blood mononuclear cells (PBMC) that can present virus-specific peptides to both CD4+ and CD8+ T cells and thus allows direct quantitation of virus-specific memory T cells. Here, we measured immune responses after smallpox vaccination as a well-characterized and robust model to determine the persistence of virus-specific T cell memory and antibody responses following childhood vaccination among HIV+ and HIV women who underwent successful immune reconstitution after antiretroviral therapy.
Methods

Subjects and study design

This is an observational 1-to-1 matched cohort study involving 50 pairs of HIV+ subjects and HIV- case controls (100 subjects, total) enrolled in the Women’s Interagency HIV Study (WIHS) (Table 1). The WIHS enrolled 4,137 women of whom 3,067 (74.1%) were HIV+ and 1071 (25.9%) were HIV- at study entry. Enrollees are followed every 6 months for interviews, physical examinations and specimen acquisition[18]. Study protocols were approved by the individual institutional review boards and informed consent was obtained from all participants (See Supplementary Methods). Inclusion criteria for HIV+ women included selection of individuals born prior to 1971 and seropositive at baseline against vaccinia virus (>200 ELISA Units), had initiated ART ≤5 years after joining the study and who had CD4+ T cell nadir <200/mm³ that improved to >350/mm³ after administration of ART. Elite controllers and seroconverters with primary HIV infection were excluded from this study to focus immunological analysis on HIV+ subjects with chronic HIV infection in which immune reconstitution was achieved by ART. Controls were matched based on age (±3 years) at the time point that T cell responses were measured (±5.5 years at time of enrollment), race, Hepatitis C virus antibody status, and were seropositive for vaccinia at baseline. Antibody decay rates were based on longitudinal serum samples (9 samples/subject in each group on average) that spanned 10-21 years of time, except for one HIV+ subject who was later found to have samples spanning only a 6-year period of time before de-enrolling from the study (median of 17.4 years of coverage for HIV+ subjects and median of 17.8 years of coverage for HIV- subjects, respectively). Subjects achieved the target CD4 count of >350/mm³ by an average of 5.6 years after entering the study (range; 1.6-14 years).

Memory T cell and antibody measurements

Cryopreserved PBMC were cultured with or without an optimized amount of sucrose-purified live vaccinia virus (multiplicity of infection = 0.3) or with anti-CD3 (Clone HIT3a (RUO), 0.05 µg/mL) as described[19] (See Supplementary Methods). Vaccinia readily infects monocytes[20] but typically does not infect primary T cells[21] (data not shown). A detection threshold of ≥20 vaccinia-specific IFNγ+TNFα+ T cells per 10⁶ T cells provides ≥95% sensitivity and ≥95% specificity[19, 22]. Serum antibodies were analyzed for 50/50 pairs of samples and viable PBMC suitable for flow cytometry analysis were recovered from 41/50 pairs. Vaccinia virus-specific enzyme-linked immunosorbent
assays (ELISA) were performed using an optimized concentration of vaccinia-infected cell lysate inactivated with 3% H$_2$O$_2$ as described[19, 23] (See Supplementary Methods).

**Statistical Analysis**

Group sample sizes of 50 and 50 achieved 80% power to detect a 4-fold difference in virus-specific CD8$^+$ T cell responses (based on preliminary data that control group mean is 8.530 with standard deviation of 3.511 in log based 2 scale) and >80% power to identify at least a 4-fold difference in virus-specific CD4$^+$ T cells (based on preliminary data that control group mean is 7.824 with standard deviation of 3.001 in log based 2 scale) and antibody levels (based on preliminary data that control group mean is 8.858 with standard deviation of 2.053 in log based 2 scale). For antibody levels, this is a conservative approach for sample size estimation because actual study design was a longitudinal study for antibody responses with 8-10 time points per subject, and a mixed effect model will be used to analyze the data. The sample size calculations and power analysis was performed using PASS 2008 software. T cell responses to anti-CD3 stimulation were compared using the non-parametric Wilcoxon Signed-rank Test. The proportion of subjects with virus-specific CD4$^+$ and CD8$^+$ T cell responses was compared using the Exact McNemar’s Test. Longitudinal ELISA data was censored[23] to remove data that dropped below the limits of detection and deemed equivocal (<200 ELISA units). Acute immune responses resulting in serospikes (i.e., doubling of ELISA titers between two contiguous points) occurred among 4/100 study subjects, resulting in an incidence rate of 0.23 events per 100 person-years. Serospikes and data from the next 3 years were removed from the analysis so that rapid decay rates that typically occur after an immunogenic event would not influence the estimated long-term decay rate. Two HIV$^+$ patients had fewer than 3 valid data points due to antibody titers below the limits of detection and they and their matched HIV$^-$ patients were not included in the mixed effects model for comparisons between groups. Rates of antibody decay were estimated using log transformed data and a longitudinal mixed-effects model. Half-life estimates were obtained by transforming the decay rate and the boundaries of the 95% confidence interval obtained from the fixed-effects slope component of the model. Analysis was performed with R3.4.4 and SAS9.4 software (See Supplementary Methods).
Results

Quantitation of memory T cells

We used intracellular cytokine staining analysis to measure the frequencies of functional CD4<sup>+</sup> and CD8<sup>+</sup> T cells among HIV<sup>+</sup> subjects at a single time point following immune reconstitution (mean: 6.4±3.1 years after ART) in comparison to HIV<sup>-</sup> case controls after direct ex vivo stimulation with anti-CD3 (Fig. 1, Table 1). This approach for T cell stimulation preferentially activates memory T cells to produce inflammatory cytokines including interferon-gamma (IFN<sub>γ</sub>) and tumor necrosis factor-alpha (TNFα) regardless of their antigen specificity. We found that 2.5% (95%CI, 1.2-3.7%) of CD4<sup>+</sup> T cells from HIV<sup>+</sup> subjects produced both IFN<sub>γ</sub> and TNFα after stimulation and that this was not significantly different from the results observed among HIV<sup>-</sup> controls (3.0%, IFN<sub>γ</sub> and TNFα, 95%CI, 2.1-4.0%, P = 0.19, Wilcoxon Signed-rank Test, Fig. 1A). Approximately 18.8% (95%CI, 15.1-22.6%) of CD8<sup>+</sup> T cells from HIV<sup>+</sup> subjects were IFN<sub>γ</sub> and TNFα after anti-CD3 stimulation. This was significantly higher than that observed among HIV<sup>-</sup> controls (13.1%, 95%CI, 10.2-16.0%, P = 0.033, Wilcoxon Signed-rank Test, Fig. 1B). Overall, cytokine profiles including IFN<sub>γ</sub> and TNFα, IFN<sub>γ</sub> and TNFα, or IFN<sub>γ</sub> and TNFα expressing T cell subsets appeared to be similar between HIV<sup>+</sup> and HIV<sup>-</sup> cohorts after anti-CD3 stimulation of CD4<sup>+</sup> T cells (Fig. 1C) or CD8<sup>+</sup> T cells (Fig. 1D). This indicates that the frequency of anti-CD3 responsive T cells among the HIV<sup>+</sup> subjects was equal to, or higher than, that observed among HIV<sup>-</sup> controls and were similar in their overall cytokine profiles.

To determine if immune-reconstituted HIV<sup>+</sup> adults maintained specific T cell memory against a viral infection that occurred prior to HIV acquisition, we examined antiviral CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses induced by childhood smallpox vaccination (Fig. 2). We have previously optimized this assay to measure vaccinia-specific T cell responses by using intracellular cytokine staining to measure dual production of IFN<sub>γ</sub> and TNFα and using a detection threshold of ≥20 vaccinia-specific IFN<sub>γ</sub> and TNFα T cells per 10<sup>6</sup> T cells provides ≥95% sensitivity and ≥95% specificity[19, 22]. Previous studies indicate that vaccinia-specific T cell memory declines with approximately an 8-15 year half-life, resulting in only a subpopulation of vaccinated individuals retaining detectable memory T cell responses when measured 20-40 years after immunization[19, 24-26]. In these current studies, the frequency of measurable vaccinia-specific memory CD4<sup>+</sup> T cells was estimated at 109 per 10<sup>6</sup> CD4<sup>+</sup> T cells (median; 59, range; 24-485 per 10<sup>6</sup> CD4<sup>+</sup> T cells) among the HIV<sup>-</sup> cohort whereas the one HIV<sup>+</sup> subject with a detectable vaccinia-specific CD4<sup>+</sup> T cell response had a score of 28 per 10<sup>6</sup> CD4<sup>+</sup> T cells – i.e., near the minimum threshold for detecting a positive T cell response (Fig. 2A). This data
indicates that 20% of HIV subjects maintained vaccinia-specific CD4\(^+\) T cell memory above the detection threshold (≥20 per 10\(^6\) CD4\(^+\) T cells) whereas only 2.4% of HIV\(^+\) subjects showed a detectable CD4\(^+\) T cell response (Fig. 2B, P = 0.039, Exact McNemar’s Test). The frequency of vaccinia-specific memory CD8\(^+\) T cells was determined within the same assays (Fig. 2C). The frequency of vaccinia-specific memory CD8\(^+\) T cells among the HIV\(^-\) cohort was estimated at 40 per 10\(^6\) CD8\(^+\) T cells (median; 34, range; 20-66 per 10\(^6\) CD8\(^+\) T cells) and this was similar to the average frequency of 61 virus-specific CD8\(^+\) T cells per 10\(^6\) CD8\(^+\) T cells (median; 32, range; 21-166 per 10\(^6\) CD8\(^+\) T cells) among the HIV\(^+\) cohort. Unlike the virus-specific CD4\(^+\) T cell responses, there was no significant difference between the percentage of HIV\(^-\) or HIV\(^+\) subjects who maintained antiviral CD8\(^+\) T cell memory (15% vs. 12%, respectively, P = 1.0, Exact McNemar’s Test, Fig. 2D). This indicates that following HIV infection and successful immune reconstitution after ART, pre-existing CD8\(^+\) T cell memory to an unrelated infection encountered during childhood (vaccinia virus) appeared to remain intact whereas pre-existing CD4\(^+\) T cell memory to the same pathogen was preferentially lost despite having normal numbers of functional CD4\(^+\) T cells in circulation (Fig. 1).

**Maintenance of serum antibodies**

HIV infection causes polyclonal B cell activation and hypergammaglobulinemia but also induces memory B cell dysfunction, exhaustion, and death[27-31]. Compared to naïve and memory B cells, much less is known about the impact of HIV and ART on plasma cell survival and the maintenance of pre-existing serum antibody responses to infections that occurred prior to HIV infection. To examine this question, we performed longitudinal analysis of antiviral antibody responses following childhood smallpox vaccination (Fig. 3A). Prior studies indicate that vaccinia-specific antibody responses are maintained essentially for life with an estimated half-life of 92 years (95%CI, 46 years-infinity)[23]. Consistent with these findings, we found that vaccinia-specific antibody responses among HIV\(^-\) subjects were stable with an estimated half-life of infinity (95%CI 309 years-infinity, Fig. 3B). In contrast, when vaccinia-specific antibody responses were measured among HIV\(^+\) subjects, they declined with a 39-year half-life (95%CI; 24-108 years) and this was significantly more rapid than HIV\(^-\) controls (P = 0.001, Fig. 3B). The 10 subjects with the most rapid antibody decay rates are shown with yellow lines in Fig. 3A. Interestingly, they were all from the HIV\(^+\) cohort and demonstrated very rapid antibody half-life estimates of between 2.6 to 8.5 years (Fig. 3B). The rapid decay rates observed among these 10 HIV\(^+\) subjects were not associated with subject age at enrollment, HBV/HCV status, initial HIV RNA viral load, residual HIV RNA viral load after ART, CD4 nadir, CD4/CD8 ratio, or time after ART before CD4\(^+\) T cell counts reached >200/mm\(^3\) or >350/mm\(^3\) (data not shown).
The rapid loss of vaccinia-specific antibodies among 20% of HIV+ women does not appear to be directly linked to loss of vaccinia-specific CD4+ T cells because the other 80% of HIV+ women appeared to have a relatively normal distribution of antibody decay rates despite the observation that all but one of these women were negative when tested for virus-specific CD4+ T cell memory (Fig. 2). We also found no correlation between antiviral antibody decay rates and CD4+ T cell memory among the HIV controls (data not shown), which is consistent with our prior studies in which no correlation between CD4+ T cell memory and antibody titers were observed among 306 vaccinia-immune adults[19]. Unlike their HIV counterparts, these studies indicate that 1 out of every 5 HIV+ subjects are prone to an accelerated loss of serological memory despite successful immune reconstitution by ART.

Conclusions

We examined the durability of antiviral T cell and antibody responses following childhood smallpox vaccination as a model to determine the impact of HIV and ART on the maintenance of pre-existing immunological memory in the absence of re-exposure or revaccination. After ART-associated immune reconstitution, HIV+ women showed no reduction in the percentage of functional, anti-CD3-responsive CD4+ T cells. However, when antigen-specific assays were employed to study immunity from smallpox vaccination, we found a nearly complete loss of virus-specific CD4+ T cell memory even though CD8+ T cell responses remained largely unchanged in comparison to HIV- controls. Analysis of vaccinia-specific antibody responses revealed a significant decline in serological memory despite successful ART-associated maintenance of peripheral CD4+ T cells. The loss of CD4+ T cell memory and antibody responses to infections encountered prior to HIV acquisition could have implications with regard to protective immunity to common acute or chronic viral infections.

CD4+ T cell-mediated cytokine responses after polyclonal anti-CD3 stimulation was not significantly different between HIV+ women or HIV- controls (P = 0.19, Fig. 1). In contrast, anti-CD3-responsive CD8+ T cell responses were significantly upregulated among the HIV+ cohort (P = 0.033). These results are consistent with prior studies in which polyclonal stimulation of peripheral T cells from HIV+ subjects showed that CD4+ T cell responses were equal to or lower than HIV- controls whereas CD8+ T cell responses were consistently higher among HIV+ cohorts[32-34]. Although the reason for increased CD8+ T cell responses among HIV+ subjects remains unclear, it is believed that immune activation may be due to a decreased ability to control repeated or chronic viral infections, resulting
in a state of persistent inflammation and an “inflammaging” phenotype[3]. In our hands, vaccinia virus-specific memory CD8⁺ T cell responses observed among HIV⁺ women appeared to be similar to that observed among HIV⁻ women in terms of the overall magnitude of the remaining memory T cell response per subject (Fig. 2C) and in terms of the proportion of subjects who maintained a detectable CD8⁺ memory T cell response (Fig. 2D). One challenge with interpreting these studies is the low frequency of T cell memory identified at late time points examined decades after acute vaccinia infection[19] and more studies are needed in order to determine if the persistent inflammation and “inflammaging” phenotype observed among HIV⁺ subjects might contribute to a more stable frequency of pre-existing CD8⁺ memory T cells or if the increased frequency of CD8⁺ T cells with a functional memory phenotype (Fig. 1B) is due to recruitment of new T cells into the memory T cell pool.

Prior studies have indicated that antigen-specific T cell memory after HIV acquisition and subsequent administration of ART was either lost[5-7] or restored[5, 8-10]. In some cases, the restoration of antigen-specific T cell responses is likely due to antigenic re-exposure after ART (e.g., cytomegalovirus, herpes simplex virus, Candida albicans, etc.)[5, 8, 9]. In one study, lymphoproliferative responses to tetanus toxoid were restored regardless of booster immunization[10]. In another study[9], lymphoproliferative responses to PPD, influenza, and tetanus toxoid remained persistently weak even after ART. CD4 nadir before ART may influence immune reconstitution since vaccine-induced CD4⁺ T cell memory did not recover among HIV⁺ subjects with a low CD4 nadir of ≤350/mm³ whereas subjects with CD4 counts remaining above 350/mm³ had memory CD4⁺ T cell responses that remained intact[6]. In our studies, we examined the recall responses to vaccinia virus antigens that are unlikely to be encountered after cessation of routine smallpox vaccination among civilians born after 1972. We enrolled HIV⁺ subjects who had CD4 nadir <200/mm³ that rebounded to >350/mm³ after ART and found that CD4⁺ T cell memory was lost among HIV⁺ subjects whereas antiviral CD8⁺ T cell memory remained intact (Fig. 2). Since CD8⁺ T cell responses were determined in the same assays as the CD4⁺ T cell responses, this indicates that differences between groups are unlikely to be due to any technical issues or cohort effects and instead indicates that CD8⁺ T cell memory is preferentially retained over CD4⁺ T cell memory after HIV acquisition and ART.
Poor antibody responses to vaccination among HIV+ individuals have been well-described[28, 35, 36]. Much less is known about the impact of HIV/ART on the maintenance of pre-existing humoral immunity. In one study, antibody responses to tetanus were maintained among 7 HIV+ subjects on ART with an average 11 year half-life[11], similar to previous studies of the general population[23, 37]. Differences in antibody decay rates may not have been observed in this small cohort if the most rapid antibody decay rates occur among only 20% of the HIV+ population as observed in our study (Fig. 3). Alternatively, the antibody decay rates could be different for particular virus or vaccine antigens. One study observed a non-significant trend towards more rapid measles antibody decay rates during primary HIV infection[12] but when monitored longitudinally during the chronic phase of infection, the antibody responses appeared stable. However, these longitudinal studies measured just a 24-month span of time, making it difficult to identify broader differences in long-term antibody maintenance. Further studies are needed to determine if rapid loss of serological memory among HIV+ subjects is unique to specific viruses/vaccine antigens or if it represents a more global defect in immune memory among these individuals. For instance, the overall rate of herpes zoster from varicella zoster virus (VZV) among HIV+ adults is nearly 4-fold higher than that observed in the general US population[2]. HIV infection is also associated with higher rates of virus-related cancers including Kaposi sarcoma (human herpesvirus-8), lymphomas (Epstein-Barr virus), anal cancer (human papillomavirus), and liver cancer (hepatitis B and hepatitis C virus). In contrast, there is no association between HIV infection and an increased risk of non-virus-associated cancers such as breast, prostate, or colorectal cancer[3]. After the introduction of HAART, the incidence of certain virus-associated cancers such KS and NHL decreased whereas cervical cancer incidence remained largely unaltered[38], indicating that prolonged immune suppression plays a role in susceptibility to some pathogens but may not completely explain the increased risks associated with HIV infection.

The loss in pre-existing serological memory among HIV+ subjects is likely due to the loss of long-lived antibody-secreting plasma cells[39], most of which reside in the bone marrow. Bone and bone marrow abnormalities that may occur after HIV/ART include osteoporosis, osteopenia, osteomalacia, osteonecrosis, low bone marrow density, and increased risk of fractures[40]. In addition, HIV infection leads to depletion of hematopoietic progenitor cells[41] and senescence of bone marrow mesenchymal stem cells, resulting in reduced support of hematopoietic stem cells in vitro[42]. HIV infects bone marrow stromal cells and HIV gp120 and Gag p55 have been shown to be involved with bone disorders[40]. Moreover, HIV therapies involving tenofovir disoproxil fumurate and protease inhibitors have been directly associated with bone abnormalities[40]. It is possible that HIV, ART, or
a combination of the two may lead to disruption of the bone marrow microenvironment needed to sustain plasma cell survival and long-term antibody responses.

This study has several limitations. We examined immunological memory among HIV+ individuals who had CD4 nadir of <200 cells/mm³ that reconstituted to >350 cells/mm³ following ART. While these levels seem adequate to describe a population experiencing severe immunologic damage and significant recovery, additional studies will be needed to determine if those with higher nadir or those with greater recovery (i.e. >500/mm³) are likely to demonstrate loss of CD4+ T cell memory (Fig. 2) or rapid decline in pre-existing antibody responses (Fig. 3). As with any long-term cohort study, a proportion of subjects are lost to follow-up due to death or attrition and it is unknown if this may impact study results. In addition, this initial study focused on HIV+ women and further studies among HIV+ men are warranted.

Despite effective immune reconstitution with ART, the loss of immunological memory to prior infections/vaccinations may play a previously overlooked role in chronic inflammation and “accelerated aging” observed among HIV+ individuals[3]. These data suggest that despite successful use of ART, HIV infection is associated with a significant loss in virus-specific CD4+ T cell memory and antiviral antibody responses that may leave a sizeable proportion of HIV+ people at increased risk for virus-associated disease manifestations.
Acknowledgements
We thank Byung Park for initial power analysis/sample size calculations and Bin Liu for help with WIHS sample acquisition. Data in this manuscript were collected by the Women’s Interagency HIV Study (WIHS). The contents of this publication are solely the responsibility of the authors and do not represent the official views of the National Institutes of Health (NIH).

Author Contributions
MKS and MHA designed the study and SH was the study coordinator. AT and EH performed the T cell and ELISA assays and AT prepared the figures, table and methods. LG performed the statistical analysis. KGM, MG, MCV, ETG, NRR, ALF, SH, and MHA contributed to the retention of participants and acquisition of samples from participants in the WIHS cohort. MKS and MHA wrote the manuscript and all authors reviewed the manuscript prior to submission.
REFERENCES


Table 1. Demographics and clinical characteristics of WIHS cohort subjects

<table>
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<th>HIV-Pos Subjects (N=50)</th>
<th>HIV-Neg Subjects (N=50)</th>
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<td>Median age, years (±SD) at enrollment</td>
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<td>% CD8^+ T cells in PBMC (±SD)</td>
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<td>Intravenous drug use</td>
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<td>15</td>
</tr>
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<td>Transfusion</td>
<td>1 (2)</td>
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Mean age at the time of T cell assay is 45±6 years and median age is 45±6 years for HIV-Pos subjects. The mean age at the time of T cell assay is 47±6 years and median age is 47±6 years for HIV-Neg subjects.

The mean and median years on antiretroviral therapy (ART) is calculated with reference to the number of years prior to the time point in which PBMC were drawn to perform the T cell assays.

Abbreviations: HBV; hepatitis B virus, HCV; hepatitis C virus, PBMC; peripheral blood mononuclear cells, SD; standard deviation, NA; not applicable.
Figure Legends

Figure 1. Cytokine production by CD4+ and CD8+ T cells after polyclonal anti-CD3 stimulation

Panel A (CD4+ T cells) and Panel B (CD8+ T cells) show the frequency of functional memory T cells from HIV+ and HIV- subjects that respond directly to anti-CD3 stimulation by simultaneously producing two antiviral cytokines, IFNγ and TNFα as determined by intracellular cytokine staining and flow cytometry. The average frequency of responsive T cells is plotted with error bars representing 95%CI. Panel C (CD4+ T cells) and Panel D (CD8+ T cells) show the proportion of anti-CD3-responsive T cells that produce both IFNγ and TNFα (IFNγ+TNFα+) or that produce only IFNγ (IFNγ+TNFα-) or only TNFα (IFNγ+TNFα-′) after direct ex vivo stimulation with anti-CD3. All values are background subtracted (medium alone) and P values were determined by Wilcoxon Signed-rank test.

Figure 2. Quantitation of vaccinia virus-specific CD4+ and CD8+ T cell memory

The quantitation of vaccinia virus (VV)-specific CD4+ T cells (A) and the percentage of HIV+ and HIV- subjects with detectable VV-specific CD4+ memory T cells (B) was determined after direct ex vivo stimulation with vaccinia virus. The frequency of VV-specific CD8+ T cell responses (C) and the percentage of HIV+ and HIV- subjects with detectable VV-specific CD8+ T cells (D) was determined after stimulation with vaccinia virus in the same assays. A frequency of ≥20 virus-specific IFNγ+TNFα+ T cells per million T cells is considered a positive antiviral T cell response. P values were determined by Exact McNemar’s Test. LOD; limit of detection, VV; vaccinia virus.

Figure 3. Longitudinal analysis of vaccinia virus-specific antibody responses

Panel A shows the longitudinal antibody responses of individual HIV+ subjects (red or yellow lines) and HIV- subjects (blue lines) as a function of age. The dashed line indicates the limit of detection (LOD; 200 EU) and values below the LOD are considered equivocal and excluded from analysis. Panel B shows the estimated half-life of vaccinia virus-specific antibody responses based on the individual slopes of each longitudinal antibody response. These values were calculated using the least squares method to show the overall distribution of vaccinia virus-specific antibody decay rates (% change in antibody levels). The average estimated antibody half-life of each group is shown with error bars representing 95%CI and statistical comparisons between groups were determined using a longitudinal mixed effects model. An average of 9 serum samples per HIV+ subject and an average of
9 serum samples per HIV+ subject were examined over a median period of 17.4 to 17.8 years of time for HIV+ and HIV- subjects, respectively. Two HIV+ subjects became vaccinia virus seronegative so quickly that there were not at least 3 data points above the LOD (the minimum for accurate half-life determinations) and so their data is presented here graphically but was excluded from the overall group antibody half-life estimation and statistical comparisons. The HIV+ individuals with the most rapid loss of virus-specific antibodies are shown as yellow lines (Panel A) or yellow-filled red symbols (Panel B).

EU; ELISA units.
Figure 3

A

Vaccinia-specific ELISA Units

Age (yr)

10^5
10^4
10^3
10^2
10^1

25 35 45 55 65 75

LOD

B

% Change in Antibody Levels

HIV-Neg  HIV-Pos

P = 0.001

∞  39 yr

Infinity

Half-life (yr)

2.3 2.8 3.5 4.6 6.9 13.9