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Title : HIV and Age Do Not Synergistically Affect Age-Related T cell Markers

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Running head: T cell markers in HIV and aging

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Abstract

Introduction:

Despite major progress in controlling HIV disease through anti-retroviral therapy (ART), changes in immune phenotype and function persist in individuals with chronic HIV, raising questions about accelerated aging of the immune system.

Methods:

We conducted a cross sectional study (2005-2007) of HIV-infected (n=111) and uninfected (n=114) males from the Veterans Aging Cohort Study. All HIV-infected subjects were on ART with VL<400 copies/mL for at least three years. T cell markers were examined via flow cytometry. We evaluated the impact of HIV serostatus and age on T cell phenotypes (expressed as percentages of the total CD4+ and CD8+ T cell population) using multivariate linear regression, adjusted for smoking, alcohol, and race/ethnicity. We tested for interactions between HIV and age by including interaction terms.

Results:

Among both HIV-infected and uninfected subjects, increasing age was associated with a decreased proportion of naïve CD4+ T cells ($p=0.014$) and CD8+ T cells ($p<0.0001$). Both HIV infection and increasing age were associated with higher proportions of effector memory CD4+ T cells ($p<0.0001$ for HIV; $p=0.04$ for age) and CD8+ T cells ($p=0.0001$ for HIV; $p=0.0004$ for age). HIV infection, but not age, was associated with a higher proportion of activated CD8+ T cells ($p<0.0001$). For all T cell subsets tested, there were no significant interactions between HIV infection and age.

Conclusion:

Age and HIV status independently altered the immune system, but we found no conclusive evidence that HIV infection and advancing age synergistically result in accelerated changes in age-associated T cell markers among virally-suppressed individuals.

INTRODUCTION

Normal aging results in alterations of T cell phenotypes, with a profound decrease in naïve T cells, and an expanded pool of terminally differentiated memory cells, sometimes referred to as components of an “Immune Risk Profile” (IRP).¹ Other components of the IRP, such as a reversed (low) CD4/CD8 ratio and clonal expansion of CD8+CD28- activated T cells have been associated with an increased risk for mortality in the elderly.^{2,3}

Untreated HIV infection is typically associated with CD4+ T cell depletion, an expansion of CD8+ T cells, high levels of T cell activation, an inverted CD4/CD8 ratio, and a skewed maturation pattern of CD4+ and CD8+ T cells, with increased proportions of cells with effector phenotypes.⁴⁻⁷ These immune phenotypes additionally are associated with inflammation (e.g., elevated interleukin-6)⁸ and immunodeficiency (i.e., low CD4+ T cells). Although anti-retroviral therapy (ART) is effective in achieving viral control in most patients, individuals with chronic HIV on ART continue to show alterations in T cell subsets compared to uninfected individuals. These include persistently high levels of T cell activation compared to uninfected controls^{9,10} and skewed proportions of naïve and memory T cells.^{7,11,12} Because of the similarities among T cell phenotypes in chronic HIV and human aging, there has been a suggestion that individuals with chronic HIV infection undergo “premature immunosenescence,” a term meant to imply that HIV and aging act synergistically on the immune system.

Clinically, individuals on long-term fully suppressive ART are at higher risk for a number of inflammatory, non-AIDS conditions, including cardiovascular disease, neurocognitive decline, osteoporosis, renal disease, and certain malignancies, compared to uninfected individuals.^{13,14} With these findings, many have speculated that HIV may therefore “accelerate” the process of immune aging as a mechanism to explain the higher incidence of these non-AIDS events compared to the general population, although epidemiological evidence has been presented

that contradicts this hypothesis.^{13,15} However, studies specifically addressing the interaction of both HIV and age on the immune system have not been systematically conducted.

Characterization of the relationship between immune cell phenotypes in the general population and age are generally limited to the study populations of the very elderly (those in their 9th and 10th decade of life).^{16,17} Recent data suggest that a subset of the population in their 7th decade of life also exhibits some of these changes, but the impact of these immune changes on clinical events has not been studied.^{17,18} Little is known about how, or in what trajectory, changes in immune phenotypes occur over a broad age range. Moreover, studies of immune aging in HIV-infected individuals have often failed to account for the presence of comorbidities in the HIV population, such as smoking and alcohol use, that might account for higher rates of age-related chronic diseases, and may also impact the immune system.¹⁹ This study broadly explored the impact of HIV and age on T cell immune phenotypes in a large cohort of HIV-infected and uninfected adults over a wide range of ages, and asked whether HIV and aging are synergistically associated with persistent changes in T cell phenotypes.

METHODS

Study design and subjects

A cross sectional analysis of HIV-infected (n=111) and uninfected (n=114) male adults from the Veterans Aging Cohort Study (VACS) was performed. VACS is a national, longitudinal, prospective, multi-site observational study of HIV-infected Veterans seen in Veterans Affairs (VA) Medical Center infectious disease clinics, along with sex, age, race/ethnicity, and site matched uninfected controls from VA general medicine clinics. Peripheral blood mononuclear cells (PBMCs), plasma, serum, and DNA were collected from a subset of VACS patients and cryopreserved. All subjects consented to the use of their specimens for future virologic,

immunologic, and genetic studies. Samples utilized for this study were obtained between 2005 and 2007. The Institutional Review Boards of the Veterans Affairs Connecticut Healthcare System, University of California San Francisco, and the Yale University School of Medicine approved this research.

PBMCs were analyzed from HIV-infected subjects who met the following inclusion criteria: HIV seropositivity for at least three years prior to initiation of antiretroviral therapy (ART); CD4+ T cell nadir <350 cells/mL prior to initiation of effective ART; and taking ART (with HIV plasma load <400 copies/mL documented at least twice a year for three years, and no HIV plasma load >1000 copies/mL in the three years prior to enrollment). Uninfected controls for this study were matched by age, alcohol use, and smoking. Alcohol use was assessed using VACS survey questions on prior and current alcohol use, and smoking history was defined by the Veteran's Affairs Health Factors dataset.²⁰ Exclusion criteria for both HIV-infected and uninfected individuals were the following: use of immunosuppressive medication in the six months prior to blood draw; diabetes mellitus (HgbA1c >6.5); renal failure (glomerular filtration rate <50cc/hr); splenectomy; malignancy; history of interferon-gamma, interferon-alpha, IL-2, or growth hormone therapy; history of bone marrow transplant; and chronic hepatitis C virus (HCV) infection (positive HCV RNA laboratory test). Cytomegalovirus (CMV) seropositivity was measured by quantitative measurement of IgG.

Cytokine flow cytometry

All subjects had cryopreserved PBMCs analyzed for T cell markers by multiparameter flow cytometry. Markers of T cell activation, T cell exhaustion, naive/memory subsets, and T regulatory cells were enumerated. After thawing, PBMCs were re-suspended in RPMI 1640 medium supplemented with 15% fetal calf serum. Flow cytometry methods are well established and detailed in prior publications, but briefly described here.²¹ Cells were washed in phosphate-buffered saline (PBS) containing 2 mM EDTA and then with PBS containing 1% bovine serum

albumin (Sigma-Aldrich). A live-dead marker was used to discriminate between live and dead T cells with two probes that measure recognized parameters of cell health: plasma membrane integrity and intracellular esterase activity (Live/Dead Viability/Vitality Kit, Molecular Probes). Cells were stained for 20 minutes at room temperature in the dark with panels of fluorescently labeled antibodies against a variety of cell surface markers in three different panels: anti-CD3-Alexa700 (BD Biosciences), anti-CD4-Pacific Blue (BD Biosciences), anti-CD8 PE-Cy55 (BD Biosciences), anti-CD25-PECy7 (BD Biosciences), anti-CD127-PE (BD Biosciences), anti-CD38-PE (BD Biosciences), anti-HLA-DR-PECy7 (BD Biosciences), anti-CD27-APC-Cy7 (BD Biosciences), anti-CD28-APC (BD Biosciences), anti-CD-CD45RA-PECy7 (BD Biosciences), and anti-CD57-FITC (eBiosciences). After fixation and permeabilization (FoxP3 fix/perm kit, eBiosciences), cells in the T regulatory cell panel were stained with anti-FoxP3-APC (eBiosciences) for 45 minutes at room temperature in the dark. Data were collected on an LSR II (BD Biosciences) flow cytometer and analyzed using FlowJo software (FlowJo LLC, Ashland, OR). For each sample, staining by the live-dead marker was used to eliminate dead/dying cells from further analysis. See Figure 1 for representative flow cytometry gating strategy.

Statistical Analysis

Frequency distributions and medians (interquartile ranges [IQRs]) are used to present descriptive statistics. Our T cell phenotype outcome variables were expressed as percentages of the total CD4+ and CD8+ T cell population, respectively: percentage of naïve (CD27+ CD28+ CD45RA+) CD4+ and CD8+ T cells, respectively; percentage of effector memory (CD27- CD28- CD45RA-) CD4+ and CD8+ T cells, respectively; percentage of activated (CD38+ HLA-DR+) CD4+ and CD8+ T cells, respectively; percentage of regulatory (CD25+FoxP3+CD127-) CD4+ T cells; and percentage of senescent (CD57+) CD8+ T cells. CD4+/CD8+ T cell ratio was examined among HIV-infected subjects only. The impact of HIV and age on T cell phenotypes was evaluated using multivariate linear regression models, adjusted for smoking, alcohol, and

race/ethnicity. The presence of an interaction between HIV and age was assessed by including an interaction term. The effect of age on T cell phenotypes in the HIV-infected group was further evaluated by adjusting for the above covariates as well as current CD4+ T cell count, nadir CD4+ T cell count, and duration of viral suppression. The assumption of normality was checked and outcomes with skewed distributions were natural-log transformed. Adjusted regression coefficients were presented as effect sizes.

For age, effect sizes are presented as per 10 years. Age effect sizes for outcomes that were not log-transformed are additive: for example, an effect size of 2% for a given T-cell phenotype means that for every 10 year increase in age, 2 percentage points are added to the percentage for that phenotype. On the other hand, age effect sizes for log-transformed outcomes are multiplicative: for example, an effect size of 1.2 for a given T-cell phenotype means that for every 10 year increase in age, the percentage for that phenotype is multiplied by 1.2. Similarly, for outcomes that were not log-transformed, an HIV effect size of 2% for a given T-cell phenotype means that the percentage for that phenotype is 2 percentage points higher for HIV-infected versus uninfected subjects. For outcomes that were log-transformed, an HIV effect size of 1.2 for a given T-cell phenotype means that the percentage for that phenotype is 1.2 times higher for HIV-infected versus uninfected subjects.

All analyses were conducted using SAS software, Version 9.4 of the SAS System for Windows (Cary, NC, USA). P values less than 0.05 were considered to be statistically significant.

RESULTS

All subjects were male. The median age was 55 years (IQR 55-61) among the HIV-infected and 55 years (IQR 48-64) among the uninfected (Table 1). HIV-infected and uninfected subjects were similar with respect to race/ethnicity, smoking, and alcohol use. Although our

intent was to exclude subjects with chronic HCV infection, we later found that two uninfected subjects had chronic HCV infection. The prevalence of CMV seropositivity was 96% among the HIV-infected and 79% among the uninfected ($p < 0.0001$). Among HIV-infected subjects at the time of blood draw, 104 patients were receiving regimens containing nucleoside reverse transcriptase inhibitors, 43 containing protease inhibitors, and 70 containing non-nucleoside reverse transcriptase inhibitor.

HIV-infected subjects had a median duration of viral suppression of 1744 days prior to enrollment (IQR 1198-2341). The median CD4⁺ count at the time of enrollment was 566 cells/mm³ (IQR 378-769), with a median CD4/CD8 ratio of 0.68 (IQR 0.45-0.95). Median CD4⁺ nadir was 167 (IQR 40- 293).

After adjusting for race/ethnicity, smoking, and alcohol use, age had a significant impact on naïve CD4⁺ and CD8⁺ T cells. For every 10 year increase in age, the proportion of naïve CD8⁺ T cells decreased by 6.3 percentage points ($p < 0.0001$) and the proportion of naïve CD4⁺ T cells decreased by 2.7 percentage points ($p = 0.014$) (Table 2; Figure 2a-b). HIV infection did not have a significant effect on levels of naïve CD4⁺ or naïve CD8⁺ T cells.

Furthermore, age was associated with a rise in the proportion of effector memory cells. For every 10 year increase in age, the proportion of effector memory CD8⁺ T cells rose by 0.24 percentage points ($p = 0.0004$) (Table 2; Figure 2). To maintain normality, the proportion of effector memory CD4⁺ T cells was log transformed; for every 10 year increase in age, this proportion increased 1.32 fold ($p = 0.041$). The proportion of effector memory CD8⁺ T cells was higher by 0.49 percentage points ($p = 0.0001$) and the proportion of effector memory CD4⁺ T cells was 2.86 fold higher ($p < 0.0001$) in HIV-infected subjects compared to uninfected controls.

To maintain normality, the proportions of both activated CD4⁺ and CD8⁺ T cells were log transformed. There was no significant effect of age on levels of activated CD4⁺ or CD8⁺ T cells. The proportion of activated CD8⁺ T cells was 1.4 fold higher in HIV-infected subjects

compared to uninfected controls ($p < 0.0001$) (Table 2; Figure 2). However HIV infection had no significant effect on levels of activated CD4+ T cells.

There was no significant effect of age or HIV infection on the the proportion of CD4+ T regulatory cells or on the proportion of senescent CD8+ T cells (Table 2; Figure 2).

Importantly, for each T cell phenotype measured, there was no significant interaction between age and HIV infection. Although the crossing of the modeled trend lines for naïve CD4+ T cells and for CD4+ T regulatory cells suggested the possibility of interactions, the p-values for interaction (0.10 and 0.18, respectively) were not statistically significant.

Additional analyses were performed to determine whether increased age continued to drive changes in T cell proportions in virally suppressed HIV-infected individuals, after further adjusting for current CD4+ T cell count, nadir CD4+ T count, and duration of viral suppression (Table 3). After further adjustment for these variables, the effect of age remained significant for naïve CD4+ and CD8+ T cells and for effector memory CD8+ T cells, but not for effector memory CD4+ T cells.

DISCUSSION

The introduction of potent combination ART has increased the lifespan of HIV-infected individuals by decades. However, there is clear evidence that patients with well-treated HIV suffer from increased rates of cardiovascular, renal, hepatic, neurologic, and bone disease, as well as certain cancers.²²⁻²⁵ This observation, in conjunction with evidence of persistent changes in immunologic parameters in ART-suppressed individuals, has led to the hypothesis that HIV infection causes premature aging of the immune system.^{26,27} To address this hypothesis, we assessed whether age and HIV infection act synergistically to impact changes in T cell subsets in 111 subjects with well-controlled HIV infection and in 114 well-matched uninfected controls.

Our study uniquely represents a large study population that evaluates T-cell phenotypes in the context of HIV status and age, but also includes a wide age range of subjects with diverse race/ethnicities and a well-matched uninfected sample.

Our study found no significant interaction between HIV and age in their effect on any of the T cell phenotypes examined, each of which has been associated with age-related changes in the general population. Thus, our study found no conclusive evidence of an increased rate of aging of the adaptive immune system among durably virally suppressed HIV-infected individuals. There was no conclusive evidence that the relationship between age and proportion of a given T cell phenotype was different among HIV-infected and uninfected subjects, and there was no conclusive evidence that the relationship between HIV status and the proportion of a given T cell phenotype was different across all ages.

Although we identified no significant interactions, we did find non-interactive independent effects of age and HIV on proportions of T cell subsets. We found that both advanced age and HIV infection were associated with increased proportions of effector memory CD4+ and CD8+ T cells, and that advanced age, but not HIV, was associated with decreased proportions of naïve CD4+ and CD8+ T cells. Furthermore, HIV, but not age, was associated with increased CD8+ T cell activation. Taken together, these findings suggest that age and HIV status independently affect the immune system, with both age and HIV associated with progression from naïve to mature T cell phenotypes and HIV associated with T cell activation.

Our finding that age is a strong predictor of the loss of naïve T cell subsets, both CD4+ and CD8+ is not unexpected, given that we know that thymic involution occurs in late childhood and continues through life resulting in decreased naïve T cell output.²⁸ Our study estimates a drop in naïve CD4+ and CD8+ T cells of 2.7% and 6.3% per decade respectively. Some have also suggested that HIV directly infects cells within the thymus and bone marrow, thus impacting naïve T cell output.^{29,30} In this study, naïve CD4+ T cells proportions did appear to have a more

pronounced decline with age among HIV-infected versus uninfected, although this difference did not achieve statistical significance. Since this would be the expected direction of any difference, it is possible that a significant interaction would have been observed with a larger sample size. It is also possible, however, that as HIV patients remain suppressed for even longer periods, this difference may be further diminished.

With regards to highly differentiated (effector memory) cells, as expected, our study finds that both age and HIV result in expansion of these T cell subsets. HIV increases the proportion of effector memory T cell subsets at every age. The expansion of effector memory T cells occurs due to continual and varied exposure to pathogens over a lifetime. In the setting of HIV infection, continuous exposure to HIV itself and to other co-pathogens results in a more dramatic expansion of effector memory cells³¹. Importantly, our study shows that although HIV-infected individuals have higher proportions of differentiated CD8+ T cell subsets at every age, there is no age-related acceleration of the rate of increase in effector T cell subsets among HIV-infected subjects.

HLA-DR and CD38 are classic markers of T cell activation that have been robustly studied in HIV-infected individuals and associated with all cause mortality in the setting of untreated HIV infection, but with still unclear impact in the setting of treated HIV.³²⁻³⁵ In patients on ART, T cell activation persists, despite successfully suppressed viremia, and is an important surrogate marker for other immunologic derangements (like low CD4/CD8 ratio), which are also associated with increased mortality.^{36,37} Our analysis reveals that HIV-infected individuals have higher levels of CD8+ T cell activation at every age, but that age itself is not associated with changes in T cell activation. Furthermore, the CD4/CD8 ratio is also not impacted by age among HIV-infected individuals.

Over the course of normal aging, CD8+ T cells undergo replicative senescence, a process through which a subset of CD8+ cells lose the ability to proliferate while maintaining an

ability to release cytokines.³⁸ This state of senescence is associated with significant changes in gene expression and cell function. Replicative senescence is thought to be driven by chronic antigen stimulation, including chronic CMV and chronic HIV infection. In this well-treated HIV population, there was no significant impact of HIV or age on CD8+ T cell senescence. Furthermore, despite an increased prevalence of CMV seropositivity among the HIV-infected, the relationship between age and proportion of senescent CD8+ T cells did not differ by HIV status.

CD4+ T regulatory cells are known to be increased in chronic infection as a response to activated state of T cells. However, in this cohort of durably virally suppressed individuals, neither age nor HIV serostatus affected the proportion of CD4+ T regulatory cells. This proportion did appear to increase with age among the HIV-infected and to remain stable with age among the uninfected, but this interaction was not statistically significant ($p=0.18$). It is possible, however, that a true interaction may have been masked by insufficient sample size or that this difference would be further diminished with longer duration of viral suppression and with more potent regimens.

A criticism of prior studies that have addressed the impact of age on immune parameters in HIV-infected and uninfected subjects is that they have compared subjects from independent cohorts without matching for potentially confounding factors, like substance use or viral co-infection.²⁷ In addition, some prior studies have relied on categorization of HIV-infected subjects into “young” and “old,” without knowledge of baseline expected rate of change in immune parameters. Our study draws from a large cohort, allowing for analysis of immune parameters along a continuum of ages. Furthermore, prior studies on aging of the immune system in the general population have typically focused on highly selected cohorts of extreme age, thus potentially missing subtle trends in phenotypic changes in T cells during the course of normal aging.^{3,39} The strength of this study of HIV and aging is that the HIV-infected and uninfected

individuals are from the same cohort, carefully matched not only for age and other demographic factors, but for also co-infections and lifestyle factors. Thus, by using a large well-matched cohort, we provide the first evidence that HIV and age do not act synergistically to cause phenotypic changes in T cell parameters among patients with well-treated HIV infection. We also provide evidence that only certain T cell subsets are impacted by age (regardless of HIV status), whereas others are impacted by HIV infection at every age.

Our study has several limitations. This was a cross sectional study, so we can not be certain that the aging-related effects we observe would be preserved if individuals were followed longitudinally over time. However, a longitudinal study over decades would be technically and logistically challenging. Our study may also be subject to a “survivor bias,” whereby the oldest subjects in the HIV-infected group may have had immunologic features that enabled them to better survive the pre-ART era. Furthermore, our study was restricted to males, so our results may not be generalizable to females.⁴⁰ In addition, although this was the largest study to date of the impact of age and HIV on T cell phenotypes with well-characterized control cohort, its statistical power to detect interactions was limited, so even larger studies would be warranted in the future, especially to study naïve CD4+ T cell and T regulatory cell proportions. In addition, future studies should explore longer durations of viral suppression, which might further diminish any differences between HIV-infected and uninfected. Finally, it is possible that age and/or HIV affect T cell subsets not examined in this investigation.

Despite these limitations, this study is the first to examine T cell phenotypes over a broad age range in individuals with well-controlled HIV infection compared to uninfected comparators, with the conclusion that well-controlled HIV does not appear to accelerate immunologic aging.

Figure Titles and Legends:

Figure 1. Gating of T cell populations. PBMCs were stained and captured by LSRII and analyzed using FlowJo software. Lymphocytes were identified by use of singlets and scatter gating. Dead/dying cells were excluded from further analysis using a viability dye. T cells were further defined by CD3 expression, and subsequently as CD4⁺ or CD8⁺ T cells. The CD4⁺ and CD8⁺ T-cell populations were each divided into subpopulations based on their expression of CD45RA, CD27, and CD28 with individual subsets identified using Boolean gating.

Figure 2. Modeled relationship between age and T-cell phenotypes, by HIV status. Black = HIV-infected, Red = uninfected. The impact of HIV and age on T cell phenotypes was assessed using multivariate linear regression models, adjusted for smoking, alcohol, and race/ethnicity. To maintain normality, outcomes with skewed distributions were log transformed. Y-axis units are percentages. Dots and lines represent modeled point estimates. Shaded gray areas represent 95% confidence bands for HIV-infected; shaded blue areas represent 95% confidence bands for uninfected. “p” indicates the p-value for interaction between HIV and age.

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