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Analyses of Mitochondrial DNA and Immune Phenotyping Suggest Accelerated T-cell Turnover in Treated HIV

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Abstract

Background: HIV infection is associated with premature aging and mitochondrial integrity is compromised during the aging process. Since mitochondrial toxicity is a consequence of antiretroviral therapies (ART), we hypothesized HIV and long-term ART would correlate with immunosenescence and mitochondrial DNA (mtDNA) pathology.

Setting: Thirteen older HIV-infected individuals (age >40 years) with virologic suppression (stratified by duration of ART) were compared to ten uninfected controls well-matched for age.

Methods: Peripheral blood T-cells were immunophenotyped to measure immune activation, proliferation, and immunosenescence in subsets. MtDNA copies/cell and the relative abundance of mtDNA carrying the “common deletion” (RACD) were quantified by droplet digital PCR.

Results: Immune activation was higher in HIV-infected individuals than uninfected in mature $CD4^+$ T-cell subsets ($CD4^+T_{TM}$ p=0.025, $CD4^+T_{EM}$ p=0.0020) regardless of ART duration. Cell populations from uninfected individuals were more likely to be more senescent populations in mature $CD4^+$ T-cell subsets (T_{TM} p=0.017), and $CD8^+$ ($CD8+T_{EMRA+}$ p=0.0026). No differences

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were observed in mtDNA or RACD levels in any $CD4^+$ T-cell subsets, while $CD8^+T_{SCM}$ of infected individuals trended to have more mtDNA (p=0.057) and reduced RACD (p=0.0025).

Conclusion: HIV-infected individuals demonstrated increased immune activation, but reduced senescence in more mature T-cell subsets. Increased mtDNA content and lower RACD in $CD8^+T_{SCM}$ suggest immune activation driven turnover of these cells in HIV-infected persons.

Keywords

immunosenescence; mitochondrial DNA; HIV; aging; common deletion

Introduction

Case-control studies have demonstrated that HIV-infected individuals experience age-related comorbidities [e.g. diabetes, cardiovascular disease, and renal disease (ESRD)] 10 years earlier than HIV uninfected age-matched controls[1]. This premature aging has been attributed to HIV associated chronic immune activation, persistent low-grade inflammation, epigenetic changes, and direct biologic effects on aging related pathways[2,3]. However, recent work suggests that aging of HIV-infected individuals may not be as accelerated as previously thought[4,5], and the differential between infected and uninfected persons may decrease with time. These and most studies characterizing premature aging in HIV-infected individuals examined cardiovascular and metabolic complications. However, other physiologic systems may also be affected by HIV infection through inflammation or direct viral effects. The immune system is one such entity, and senescence in this system manifests as reduced breadth and potency of the immune response[6]. This results in reduced responses to vaccination[7,8], increased rates of infection[9], and reduced immune surveillance[10]. Immunosenescence is also associated with immune dysregulation that results in persistent immune activation and the senescence associated secretory phenotype (SASP), resulting in the chronic low-level inflammation seen in with aging and in HIV-infected individuals[11–13].

The pathogenesis of aging and cellular senescence is complex, but includes mitochondrial alterations within cells[13]. Damage to mitochondria is thought to play a role in many age-related pathologies, and natural again is associated with increases in mitochondrial DNA (mtDNA) mutations and declines in mitochondrial function (reviewed in [14]). We and others have previously demonstrated that mitochondrial DNA deletions accumulate with age in the brain[15,16] and in muscle[17–19]. Specifically, the proportion of mtDNA carrying the “common deletion”, a 4977bp deletion that affects several transfer RNA and respiratory chain genes has also been associated with aging related phenomena such as corneal thickening, hearing loss, loss of skin turgor, and sarcopenia[17,20–25]. If this deletion is present in a high enough proportion of the mtDNA within a cell, the cell will be incapable of meeting its metabolic demands[26]. This deletion is more common in older populations and in longer-lived cells often involved in degenerative diseases[17,27–29]. We hypothesized that since HIV infection results in cumulative mitochondrial toxicity from direct viral effects and ART[30–32], immune cells from infected individuals may have greater mitochondrial DNA pathology than immune cells from age-matched uninfected individuals. We further hypothesized that the degree of mitochondrial DNA pathology would correlate with greater immunosenescence and immune activation. To address this question, we measured immunosenescence, immune

activation and mitochondrial DNA changes in circulating T-cells from persons living with HIV older than 40 years and compared them to aged-matched HIV uninfected controls.

Materials and Methods

Study Cohort

The study sample consisted of 13 HIV-infected individuals over the age of 40 who had been on HIV therapy for greater than two years, and 10 healthy uninfected controls well-matched for age enrolled between July and December 2014. Individuals with cancer, hepatitis C, diabetes, or on immunosuppressive medications were excluded from the study. The study was approved by the Human Research Protections Program at the University of California San Diego (UCSD). All HIV-infected individuals were recruited from the UCSD Owen clinic and the UCSD Antiviral Research Center. Control subjects were recruited from UCSD general internal medicine clinics. After providing informed consent, sociodemographic and clinical data were obtained from each subject, and 100 cc and 8cc of fresh whole blood (in ACD and EDTA tubes, respectively) was collected for further analysis.

Determination of absolute T-cell counts

Absolute counts of T-cells in whole blood were determined by flow cytometry (Accuri, BD biosciences). First, the absolute lymphocyte count was determined using an CD45-PerCP-Cy.5.5 antibody in combination with perfect count microspheres (Life technologies). Then, the percentage of the T-cells was determined using the following antibody combination: CD3-APC, CD4-FITC and CD8-PE (all antibodies were from BD Biosciences). Absolute count of each cellular population was calculated as follows: (X*Y)/100, where X is the percentage of each subset and Y is the absolute count of lymphocytes.

Immunophenotyping

The levels of proliferation (Ki67 FITC, tube 1), immune activation (CCR5 FITC, HLA-DR BV421 and CD38 BV605, tube 2) and immunosenescence (CD57 FITC and KLRG1 BV421, tube 3) in $CD4^+$ and $CD8^+$ T-cell subsets were evaluated (common backbone for all tubes: CD3 APC-H7, CD4 PerCP-Cy5.5, CD8 V500, CD45RA AF700, CD27 PE-Cy7, CCR7 PE-CF594, CD58 PE and CD95 APC). Briefly, 150μl of whole blood/tube was incubated for 20 minutes at room temperature with the antibody combinations. Cells were then lysed for 10 minutes at room temperature in FACS Lysing solution (BD Biosciences), washed in PBS and fixed in PBS containing 1% formaldehyde (Sigma), before acquisition in an FACS Canto (BD Biosciences). For tube 1, cells were washed after lysis and then fixed and permeabilized using FoxP3 staining buffer set (eBiosciences) for 30min at 4°C and then incubated with anti-Ki67 for 30min at 4°C. CD4 ($CD3^+CD4^+CD8^-$) and CD8 ($CD3^+CD8^+CD4^-$) T-cell subsets were defined as: T_N:$CD45RA^+CD27^+CCR7^+CD95^-CD58^-$, T_{SCM}:$CD45RA^+CD27^+CCR7+CD95^{br}CD58^{br}$, T_{CM}: $CD45RA^-CD27^+CCR7^+$, Ttm: $CD45RA^-CD27^+CCR7^-$, $T_{EMRA}-$: $CD45RA^-CD27^-CCR7^-$ and T_{EMRA+}: $CD45RA^+CD27^-CCR7^-$. For each subset, the expression of proliferation, activation and senescence makers were evaluated.

Quantification of mtDNA within T-cell subsets

Blood from ACD tubes was immediately processed for isolation of peripheral blood mononuclear cells (PBMCs) using Ficoll–Hypaque density gradients. PBMCs were divided in two aliquots to negatively select $CD4^+$ or $CD8^+$ T-cells (Easysep CD4 and CD8 enrichment kit, Stem cell). Enriched CD4 and CD8 T-cells were then labeled (CCR7 FITC, CD58 PE, CD45RA-PE-CF594, CD3 PerCP-Cy5.5, CD27 PE-Cy7, CD95 APC and CD4 or CD8 APC-H7) for live-cell sorting (MoFlo, Beckman Coulter) to obtain populations of T_N, T_{SCM}, T_{CM}, T_{TM} and T_{EM} $CD4^+$ and $CD8^+$ T-cells (Supplemental Figure 1). Following sorting, sorted subsets were centrifuged 300g for 5 min, and the supernatants were carefully discarded. Cell pellets were resuspended in a lysis buffer (0.05% Nonidet P-40 and Tween-20 [Sigma], 0.1mg/ml proteinase K [Invitrogen]) and digested for 30min at 65°C. Proteinase K was inactivated by heating digested samples at 95°C for 15 min. Cells lysates were stored at −20°C until use. We measured the mitochondrial DNA (mtDNA) copy number per cell for each subset using droplet digital PCR (BioRad) directly form cell lysates. Quantification was performed as previously described[16] using a primer-probe set designed to target a gene on the mitochondrial genome, *MT-ND2*[33]. A second primer-probe set targeting human *RPP30* was used to as a cell-copy control, as each cell contains two copies of this gene. In addition to quantifying the total mtDNA copy number, the proportion of mtDNA carrying the “common deletion” was also measured. As previously described[16], we measured the proportion of mtDNA carrying this deletion using a primer-probe combination targeting the bridge sequence formed by the ends of the mitochondrial genome left by the common deletion[33]. This proportional measure of the common deletion was used as a surrogate measure of mitochondrial somatic damage.

Statistical Analyses

All statistical analyses were performed using the R statistical package. Normality of the levels of mtDNA and relative presence of the “common deletion” were assessed using a Shapiro test with a significance cut-off of $p<0.05$. Given that log transformation did not normalize data, analysis was performed with non-transformed data. Differences in mtDNA levels and relative presence of the “common deletion” between HIV-infected and uninfected study groups were assessed by the students t-test and by using analysis of variance (ANOVA) when comparing the individuals infected <15 years and >15 years with the uninfected controls. If data failed normality, a Kruskal-Wallis test was used to assess differences between study groups. No corrections for multiple comparisons were made given the exploratory nature of this study.

Results

Study Participant Characteristics

Thirteen virally suppressed HIV-infected individuals with a median age of 51 (Range: 42–68 years) and 13 HIV uninfected controls with a median age of 50.5 (Range: 45–72) were enrolled in the study. Data from three uninfected individuals was subpar, and not included in the analysis. HIV-infected individuals were on therapy for a median of 15 years (Range: 2–29 years). Five individuals had previous exposures to d4T, ddI, ddC, or AZT (medications known to cause mitochondrial toxicity), but no participants were currently on those

medications. The median $CD4^+$ T-cell nadir for the HIV-infected group was 175 cells/mm^3 (Range: 0–628 cells/mm^3), and there were no differences in $CD4^+$ or $CD8^+$ T-cells percentage in each subset between the infected and uninfected groups (Supplemental Figure 2). Additional characteristics are presented in Table 1.

T-cell activation in persons living with HIV compared to age matched controls

Immune activation as measured by dual expression ($HLADR^+CD38^+$) was higher in HIV-infected individuals than uninfected in the mature $CD4^+$ T-cell subsets (T_{TM} p=0.025, T_{EMRA}- p=0.0025, Figure 1A). This remained true whether the individual had been on treatment for <15 years or >15 years (T_{TM} p=0.04/p=0.09, T_{EM} p=0.07/0.003, Supplemental Figure 3A). No differences in immune activation were seen in $CD8^+$ T-cell subsets as measured by differences in $HLADR^+CD38^+$ expression (Figure 1B). Strangely, infected person with <15 years of treatment trended toward lower immune activation in Tn, Tscm and Ttm CD8+ T-cell subsets than uninfected controls (T_N p=0.06, T_{SCM} p=0.096, T_{TM} p=0.096) (Supplemental Figure 3B).

T-cell senescence in persons living with HIV compared to age matched controls

To evaluate differences in T-cell senescence between HIV-infected and uninfected individuals, we evaluated expression of KRLG1 and CD57 on subsets of $CD4^+$ and $CD8^+$ T-cells. Long-lived T_N and T_{SCM} $CD4^+$ T-cell subsets from HIV-uninfected individuals were less senescent (T_N p=0.02, T_{SCM} p=0.07). However, the T_{TM} subset was more senescent in uninfected individuals (T_{TM} p=0.02) (Figure 2A). When senescence was defined by dual expression of $KRGL1^+CD57^+$, the $CD4^+T_{TM}$ T-cell subset continued to appear more senescent in the HIV-uninfected population (T_{TM} p=0.001), while again the HIV-infected individuals had higher levels of $CD57^+KRLG1^+$ in $CD4^+T_N$ cells (T_N p=0.03) even though the numbers of cells were very low. (Supplemental Figure 4A). $KRGL1^+CD57^+$ expression differences in $CD4^+$ T-cells were more pronounced when uninfected persons were compared to HIV-infected persons treated for >15 years (uninfected vs. treated for >15 years, p=0.001) (Supplemental Figure 5A).

Regarding the $CD8^+$ T-cell compartment, HIV-uninfected individuals had higher proportions of senescent T_{EMRA}+ $CD8^+$ T-cells as measured by $KRLG1^+$ (p=0.003, Figure 2B), and $CD57^+KRLG1^+$ (p=0.007, Supplemental Figure 4B) compared to the HIV-infected individuals. The lower observed senescence of T_{EMRA}+ $CD8^+$ T-cells in HIV-infected persons did not differ when individuals were stratified by treatment duration when compared to uninfected controls (Supplemental Figure 5B).

T-cell proliferation in persons living with HIV compared to age matched controls

To evaluate differences in T-cell proliferation between HIV-infected and uninfected individuals, we evaluated expression of Ki67 on subsets of $CD4^+$ and $CD8^+$ T-cells. We found that $CD4^+$ T-cells from HIV infected individuals were more likely to express Ki67 molecule than uninfected individuals (p=0.06), and this was most pronounced in the T_{EM} population (p=0.011) (Supplemental Figure 6).

Relative mitochondrial DNA abundance in Peripheral Blood T-cells

To assess if mitochondrial toxicity contributed to differences in immune activation and senescence we evaluated mitochondrial DNA (mtDNA) copy number, measured by copies of *MT-ND2,* and the relative abundance of the mitochondrial common deletion in $CD4^+$ and $CD8^+$ T-cell subsets (Figure 3). No differences in total mtDNA in $CD4^+$ T-cell subsets were observed between infected and uninfected individuals. However, $CD4^+$ T-cell subsets demonstrated lower mtDNA copy numbers in T_{TM} than in T_{SCM} (p= 0.030) and T_{EM} (p=0.09) (Figure 3A). A similar trend was observed between T_{TM} and T_{SCM} from uninfected controls (p=0.08).

More differences were observed in mtDNA from $CD8^+$ T-cells. In HIV-infected subjects, T_{SCM} had higher levels of mtDNA than in any other subset (T_N p=0.04, T_{CM} p=0.09, T_{TM} p=0.08 and T_{EM} p=0.0002) (Figure 3B). The T_{SCM} subset also trended towards higher mtDNA levels in the HIV-infected group compared to uninfected controls (p=0.057). In contrast, uninfected individuals showed higher levels of mtDNA in T_N compared to T_{TM} (p=0.05) and T_{EM} (p=0.006), and T_{CM} showed higher levels of mtDNA than T_{EM} (p=0.002). The three subjects on treatment for the shortest period (2, 8, and 9 years) had the highest levels of *MT-ND2* detected in the T_{CM} and T_{TM} subsets of both $CD4^+$ and $CD8^+$ populations, but we did not observe any significant differences between individuals on treatment for less than or more than 15 years.

Relative abundance of mitochondrial DNA carrying the common deletion in T-cells

The relative abundance of mtDNA carrying the common deletion (RACD) did not differ between HIV-infected and HIV uninfected individuals in any T-cell subsets except for $CD4^+$ and $CD8^+$ T_{SCM} cells, where a higher RACD was seen in uninfected individuals (p= 0.06 and p=0.003, respectively) (Figure 3C and D). This difference in $CD8^+$ T_{SCM} cells, was most prominent when uninfected persons were compared to those with HIV infection >15y (p=0.002) (Supplemental Figure 7). RACD negatively correlated with *MT-ND2* only in the $CD4^+$ and $CD8^+$ T_{EM} subsets ($CD4^+$: $r^2=-0.31$, p-value=0.005, $CD8^+$: $r^2=-0.31$, p-value=0.005).

Correlation of mitochondrial DNA with senescence, activation, and proliferation markers

We did not identify any associations between average mtDNA or RACD and senescence, activation or proliferation markers in $CD4^+$ or $CD8^+$ T-cell subset populations. However, we did note that the direction of correlation between proliferation and RACD in T-cell subsets was nearly always negative, but that high RACD in mature $CD4^+$ T-cell subsets was associated with proliferation of $CD4^+T_{SCM}$ (Supplemental Tables 1 and 2)

Correlation of mitochondrial DNA with age and years living with HIV

We next evaluated the correlation between age and mtDNA content per cell. In $CD4^+$ and $CD8^+$ T_N subsets (when combining both HIV-infected and uninfected individuals), we observed a correlation between age and mtDNA copies per cell ($CD4^+T_N$: $r^2=0.25$, p=0.014; $CD8^+T_N$: $r^2=0.245$, p=0.015. Consistent with this, we also found that age correlated with the RACD in the mature subsets of both $CD4^+$ and $CD8^+$ populations ($CD4^+T_{TM}$: $r^2=0.23$,

p=0.02; $CD8^+T_{TM}$: r^2=0.22, p=0.025; $CD8^+T_{EM}$: r^2=0.28, p=0.009) (Supplemental Figure 8).

No correlations were observed between age and mtDNA in the infected or uninfected population alone. However, there was a trend towards a correlation between the number of years infected with HIV with mtDNA levels in $CD4^+T_N$ (r^2=0.29, p=0.09), and with the RACD in the $CD4^+T_{TM}$ subset (r^2=0.41, p=0.03). The time on ART also correlated with the RACD of the $CD4^+T_{TM}$ subset (r^2=0.51, p=0.014) (Supplemental Figure 8).

Discussion

This cross-sectional study is one of the first to examine the relationship of mtDNA with immunosenescence and immune activation in the setting of aging with HIV. We observed that HIV-infected individuals on suppressive ART demonstrated higher levels of $CD4^+$ T-cell immune activation than HIV negative persons particularly in the T_{TM} and T_{EM} subsets, consistent with the function of these cells and previously reported literature[34,35]. Unexpectedly, age-matched uninfected controls in our cohort had higher proportions of $KLRG1^+$ senescent T-cells in more mature $CD4^+$ and $CD8^+$ subsets. We observed this to be true for $CD57^+KLRG1^+$ $CD4^+T_{TM}$ cells as well. The seeming discordance between immune activation and immunosenescence could suggest that 1) higher levels of ongoing cellular inflammation in HIV-infected individuals do not result in terminal differentiation of lymphocytes[36–38], or 2) HIV infection and chronic immune activation may drive increased $CD4^+$ and $CD8^+$ T-cell death[39,40] and increased cellular turnover (in response to T-cell depletion) preventing the accumulation of senescent cells. This second hypothesis was supported by our observation that $CD4^+$ T-cells, (particularly the T_{EM} subset) demonstrated higher rates of proliferation than controls. The Tem subset also demonstrated an inverse relationship between mtDNA and RACD suggesting increased cell turnover. We observed higher mtDNA and a smaller RACD in the long-lived stem cell subset (T_{SCM}) of HIV-infected individuals compared to uninfected individuals, which is consistent with increased mitochondrial turnover in the setting of cellular proliferation, as suggested by Ross *et al*[41]. Although not always statistically significant, the direction of correlation between T-cell subset RACD and Ki67 expression (proliferation), and RACD and mtDNA content was always negative.

In contrast, we observed an increase in mtDNA per cell with increasing age in the long-lived T_N subset in both HIV-infected and uninfected individuals. This was most pronounced in the HIV-infected individuals with infection >15 years. The T_N population from HIV-infected individuals also had a higher proportion of $CD57^+KLRG1^+$ $CD4^+$ T_N than uninfected individuals, which may suggest a shift to reduced cellular replicative capacity. We did not identify a relationship between RACD and mtDNA content in this subset. Unfortunately, further exploration of this concept was beyond the scope of this study, but others have demonstrated an association between increased senescence and reduced mitophagy[42], which may lead to accumulation of damaged mtDNA.

This pilot cross sectional study is limited by its small sample size, and the clinical heterogeneity of the subjects (i.e. significant variations in duration of infection and time on

ART). Furthermore, we did not evaluate or measure other potential factors that could contribute to or impact inflammation and immune aging, including: smoking history, CMV status, antiretroviral regimen, measures of nuclear DNA replicative capacity (e.g. telomerase activity), cellular division (e.g. TRECs), and other intracellular physiologic processes[43,44]. Specifically, the antiretroviral exposure of individuals on treatment >15 years was more likely to include medications with a higher mitochondrial toxicity. As in other studies, measurements of mitochondrial DNA and RACD were across populations of cells, and the distribution of these measures was unlikely to be uniform. We also confined our evaluation of T-cell immune activation and immunosenescence to the peripheral blood and therefore cannot confirm that our findings were not due to differences in T-cell trafficking to effector sites. Lastly, as an exploratory study, we did not statistically correct for multiple comparisons, limiting the interpretation of our findings.

In summary, we did not observe clear and consistent evidence of immunosenescence or mitochondrial signals of aging despite higher levels of cellular immune activation in the peripheral blood of HIV-infected persons[34,35]. Our data suggest this may be due to higher cell turnover in individuals with more recent HIV infection. This is different than what we observed in our study of mtDNA in the brain, where neurons and other glial cells live for extended periods of time. Previous studies attempting to quantify the accelerated aging of HIV-infected individuals have estimated a 2–10 year increase in biologic age greater than HIV uninfected individuals of the same chronologic age[45,46]. Our study suggests that in the first few years of treated HIV infection, immune activation continues to drive turnover of T-cells, a process that appears to decrease with time on therapy. Thus, using peripheral T-cells to assess aging may underestimate biologic age, particularly in more recently infected individuals, and limits our ability to use these cells to evaluate the effects of HIV on aging. The results of this study are limited by the heterogeneity of the participant population and small sample study, and therefore further work into this area is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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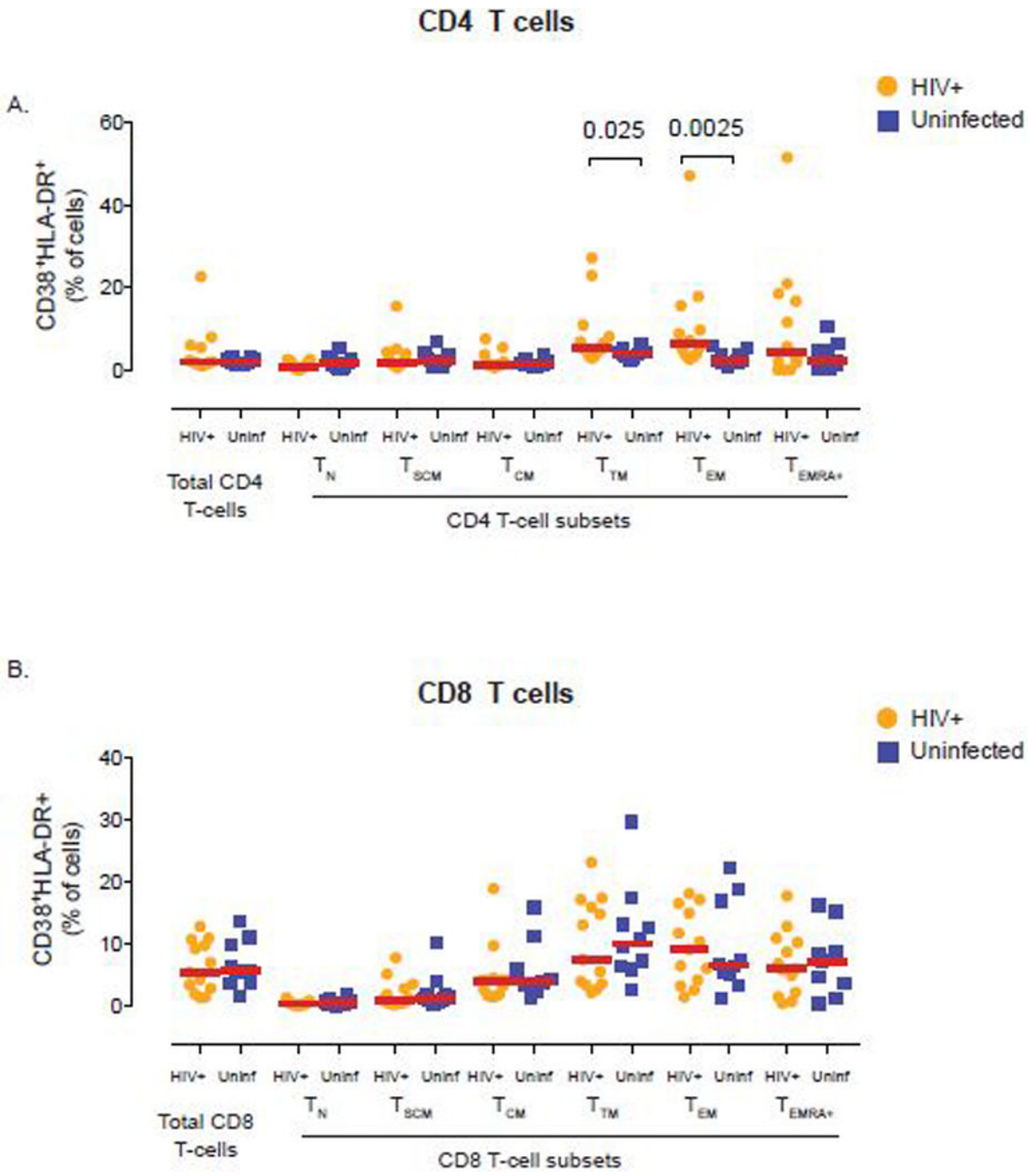

Figure 1. Immune Activation.

Immune activation measured by $CD38^+HLADR^+$ expression in $CD4^+$ (Panel A) and $CD8^+$ (Panel B) T-cell subsets. The T_{EM} $CD4^+$ T-cell subset from HIV-infected individuals was more likely to demonstrate immune activation (by $HLADR^+CD38^+$ expression). P-values between age matched infected and uninfected individuals are indicated.

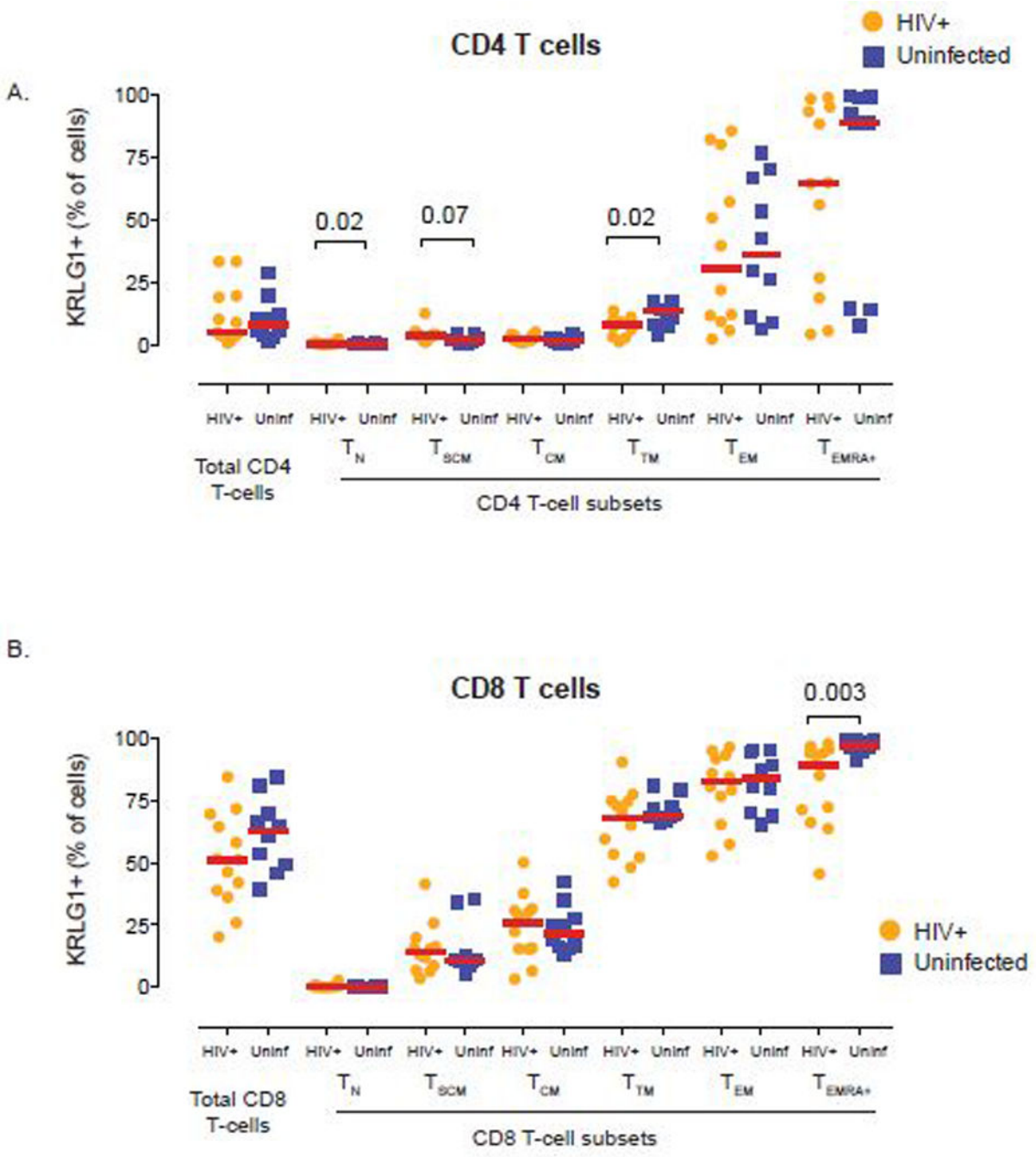

Figure 2. Senescence.

Senescence measured by KRLG1^{+} expression in CD4^{+} (Panel A) and CD8^{+} (Panel B) T-cell subsets. CD4^{+} (T_N, T_{SCM}, and T_{TM}) and CD8+ (T_{EMRA+}) T-cells from uninfected individuals were more likely to be senescent. P-values between age matched infected and uninfected individuals are indicated.

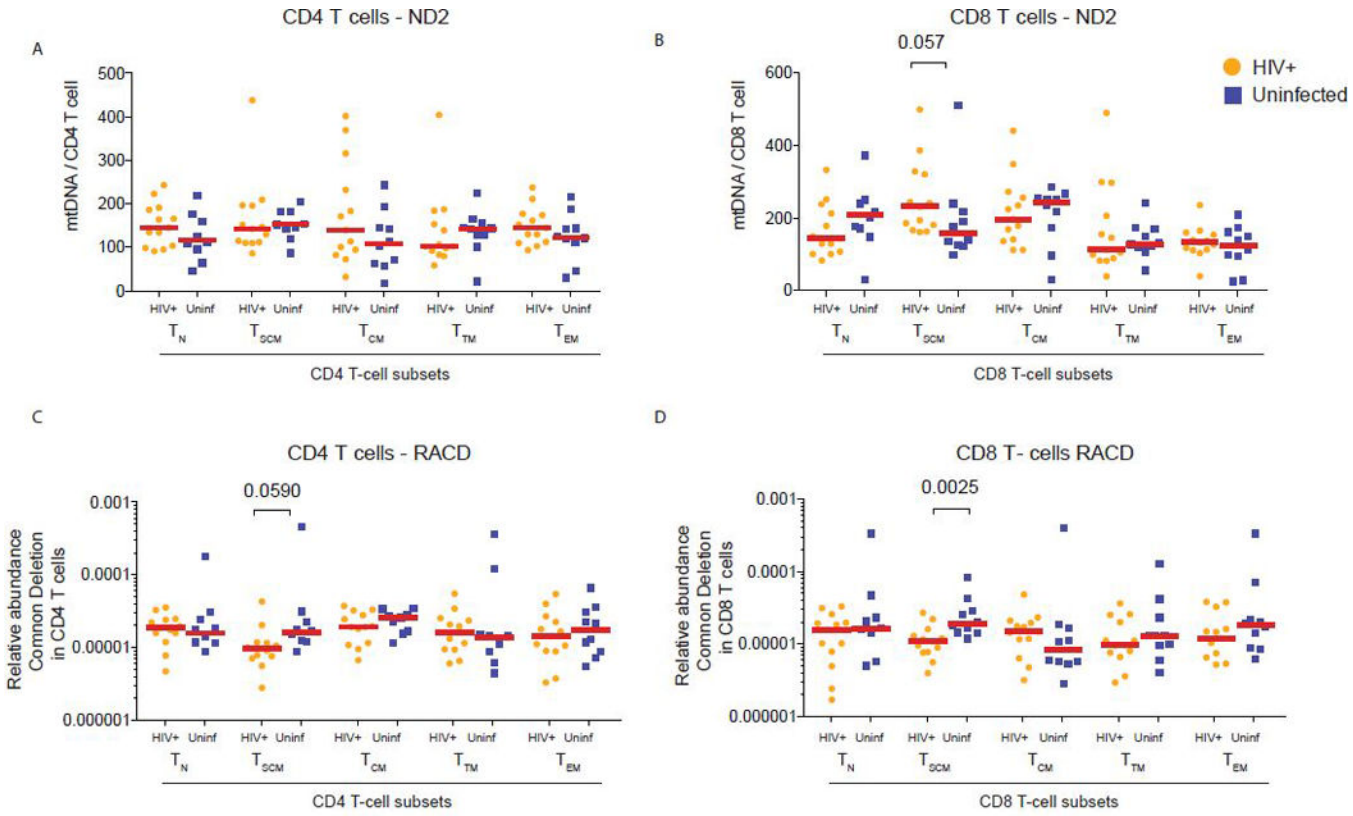

Figure 3. Mitochondrial DNA Measurements.

Mitochondrial DNA copy number (ND2) and the relative proportion of mitochondrial DNA carrying the common deletion (RACD) in $CD4^+$ (Panels A and C) and $CD8^+$ (Panels B and D) T-cells. $CD8^+$ (T_{SCM}) from infected individuals had higher mtDNA levels and lower proportions of mtDNA with the common deletion. Across other T-cell subtypes, this was not statistically significant but visually trended in that direction. Overall, this suggests increased cell-turnover in the T-cells of infected individuals.

Table 1.

Study Cohort

	HIV+ (n=13)	HIV+ <15 y (n=5)	HIV+ >15 y (n=8)	Uninfected (n=10)	P-value HIV+ vs Uninfected
Age, median[IQR]	51 [46–56]	51 [47–54]	46 [52–59]	51 [47–60]	*0.843*
Males, n(%)	13 (100)	5 (100)	8 (100)	10 (100)	*1.00*
Race, n(%)					
Whites	9 (69)	4 (80)	5 (62.5)	7 (70)	*1.00*
African-American	3 (23)	0	3 (37.5	1 (10)	*0.6*
Asian	1 (7)	1 (20)	0	2 (20)	*0.56*
Time since diagnosis, years, median [IQR]	21 [13–24]	9 [8–12]	24 [23–28]	-	-
Time on treatment, years, median [IQR]	15 [9–19]	8 [8–9]	19 [18–22]	-	-
CD4 absolute counts, cells/μl, median [IQR]	586 [409–729]	586 [408–634]	602 [425–773]	787 [708–1058]	***0.004***
CD8 absolute counts, cells/μl, median [IQR]	790 [472–1032]	900 [790–994]	592 [457–895]	439 [315–955]	*0.227*
CD4%, median [IQR]	45.5 [34.1–52.3]	35.4 [28.5–45.8]	48.1 [37.9–52.8]	60.7 [52–67.7]	***0.004***
CD8%, median [IQR]	50.5 [45.2–62.6]	59.7 [50.5–66.7]	48.8 [44.9–57.7]	37.0 [29.2–44.0]	***0.004***
Ratio CD4/CD8	0.9 [0.5–1.2]	0.6 [0.4–0.9]	1.0 [0.7–1.2]	1.7 [1.2–2.3]	***0.004***
HIV Viral Load, <50 copies/μl, n(%)	13 (100)	5 (100)	8 (100)	-	-