Mucosal-homing natural killer cells are associated with aging in persons living with HIV

Graphical abstract

Highlights

- NK cells during aging display increasing expression of gut-homing marker α4β7
- NK cells from aging PWH, regardless of treatment, upregulate lymph node-homing CCR7
- CMV status does not appear to significantly impact NK cell trafficking in PWH

In brief

Advances in ART have led to an aging population of people living with HIV (PWH). NK cells play a key role in viral control and also demonstrate distinct trafficking-marker patterns in aging. Disruption of mucosal and lymph node homing in aging PWH could have significant impacts on virus control in lymph nodes and gut homeostasis.

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Mucosal-homing natural killer cells are associated with aging in persons living with HIV

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SUMMARY

Natural killer (NK) cells are critical modulators of HIV transmission and disease. Recent evidence suggests a loss of NK cell cytotoxicity during aging, yet analysis of NK cell biology and aging in people with HIV (PWH) is lacking. Herein, we perform comprehensive analyses of people aging with and without HIV to determine age-related NK phenotypic changes. Utilizing high-dimensional flow cytometry, we analyze 30 immune-related proteins on peripheral NK cells from healthy donors, PWH with viral suppression, and viremic PWH. NK cell phenotypes are dynamic across aging but change significantly in HIV and on antiretroviral drug therapy (ART). NK cells in healthy aging show increasing α4β7 and decreasing CCR7 expression and a reverse phenomenon in PWH. These HIV-associated trafficking patterns could be due to NK cell recruitment to HIV reservoir formation in lymphoid tissue or failed mucosal signaling in the HIV-infected gut but appear to be tight delineators of age-related NK cell changes.

INTRODUCTION

Advances in combination antiretroviral drug therapy (cART) and early intervention led to HIV becoming a chronic illness and an increase in life expectancy of people with HIV (PWH). Consequently, the population of PWH are aging, with those over the age of 50 accounting for over 20% of PWH worldwide and 51% in the United States. However, the necessity of long-term ART poses a potentially serious problem as PWH age, as its impact on the immune system is still not completely understood. Chronic inflammation is suggested to be a leading factor of morbidity during aging, and PWH exhibit this chronic inflammation despite long-term viral suppression. Furthermore, aging PWH are at higher risk of age-related co-morbidities, such as cardiovascular disease, and polypharmacy is common, which can lead to potential drug-drug interactions. The progression and burden of age-related comorbid conditions and multimorbidity in people aging with HIV (PAWH) differs proportionally in several ways from the general uninfected population; however, the mechanisms and impact of viral-directed immune responses is still not completely understood.

Natural killer (NK) cells, potent innate immune cells important in viral and tumor surveillance and immunoregulation, have been shown to play a critical role in HIV. Specific killer immunoglobulin-like receptors (KIRs) and human leukocyte antigen (HLA) combinations have been shown to be highly effective at control and protection from HIV infection. Additionally, it has been shown that high NK cell functional capacity is closely associated with inhibited HIV transmission. Interestingly, in non-human primate models of simian immunodeficiency virus (SIV), NK cells are also shown to be highly plastic and undergo large shifts in trafficking to lymph nodes and/or gut mucosal sites. However, NK cells have also been shown to become exhausted during chronic HIV infection with typical signs being increased frequency of CD56dimCD16+ NK cells but a decrease in functional responses and a loss of Siglec-7 expression. Furthermore, aging has also been characterized by chronic low-grade inflammation, alterations and dysfunction in adaptive immune responses, and changes in innate immune cells. NK cells in aging show similar dysfunctions that are seen in chronic HIV infection, namely an increase in CD56dimCD16+ NK cells but a decrease in functional responses and a loss of Siglec-7 expression. In addition to an increase in raw numbers of NK cells with age, they also have reduced proliferative potential, decreased surface expression of the activating receptors Nkp30 and Nkp46, modulated KIR expression, and loss of Siglec-7 and Siglec-9 expression, an accumulation of senescent cells that may be a result of age-related decline in NK cytotoxicity, and age-related trafficking changes that are directly responsible for increased susceptibility to certain pathogens.
To understand mechanisms underlying the interactions between NK cells, aging, and HIV control, we examined NK cell phenotypic changes in PWH either on effective ART or untreated across a broad age span. We developed a high-dimensional flow cytometry comprehensive panel to measure 30 immune-parameters consisting of trafficking markers, NK cell receptors, activating/inhibitory receptors, and senescent cell markers. By defining HIV and aging-specific NK cell perturbations, this will allow for the development of novel approaches to limit or reverse innate immune dysfunction, alter trajectories of co-morbidities, and improve clinical outcomes in PAWH.

RESULTS

Study design and cohort demographics
For our analysis, we utilized a cohort of 135 samples collected by the Hawai’i Aging with HIV-1 Cohort (HAHCh) at the University of Hawai’i, comprised of healthy donors (HDs; n = 49), PWH on treatment (cART; n = 61), and PWH off treatment (viremic PWH; n = 25). Due to sample quality issues (data not shown), several samples from each group were removed from the dataset. The resulting dataset consisted of 125 samples (HD n = 46; cART n = 58, viremic n = 21). The three groups had similar age ranges, with HDs having a median age of 48.0 years (32.5–73.5), cART having a median age of 53.0 years (26.7–73.3), and viremic PWH having a median age of 42.3 years (22.3–78.1) (Table S1). Gender proportion, duration of known infection, viral load, and other clinical information can be found in Table S1. High-dimensional polychromatic flow cytometry was utilized during this study to quantify surface protein expression of a broad array of receptors. The panel designed covered proteins that broadly fell into (1) trafficking receptors, (2) activation/inhibitory receptors, (3) adaptive/memory markers, and (4) immune exhaustion markers (key resources table). Due to the known impacts of HIV on frequencies of CD56<sup>bright</sup>CD16<sup>+</sup> and CD56<sup>dim</sup>CD16<sup>+</sup> NK cells, most of our analyses focus on the CD56<sup>dim</sup>CD16<sup>+</sup> NK population due to challenges in accurately defining CD56<sup>bright</sup>CD16<sup>+</sup> NK cells in PWH. In addition, this population is dominant in circulation as well as being the cytotoxic effector population most linked with viral control.

NK cells in aging HDs show paucity of receptor repertoires
To determine the impact of HIV on NK cells during aging, we first examined HIV uninfected donors (HDs) in two age stratifications (under the age of 45, and over the age of 50) to establish a baseline “aged phenotype” of NK cells in the absence of known HIV infection. We chose to use 50 years old as the cutoff for the aged population because this has been previously described as an age to define medically advanced age in PWH. Specifically, PWH over 50 were shown to have both poorer responses to ART and poorer survival. The median age of HDs was 48.0 years old with a range of 32.5–73.5 years old. We focused our analyses on the dominant blood phenotype of cytotoxic CD56<sup>dim</sup>CD16<sup>+</sup> NK cells. Representative gating strategies for each group are shown in Figure S1, and representative single marker staining plots are shown in Figure S2. Generalized linear modeling (GLM) with bootstrap resampling was employed to examine the log odds of protein expression being a predictor of age group (Figure 1A). GLM analysis results indicated that an increase in CD127 (interleukin-7R<sub>a</sub> [IL-7R<sub>a</sub>]) expression with aging is a significant predictor of aging in HDs (p = 0.021; Figure 1A). In contrast, lower CD8<sub>c</sub> (p = 0.021) and CCR7 (p = 0.042) expression are significant predictors of younger people (Figure 1A), while KIR3DL1/S1 showed a trend toward younger individuals (p = 0.063; Figure 1A). Interestingly, CCR7 was a significant predictor (p = 0.042) and was negatively correlated with age (R = −0.5219, p = 0.0005; Table S2), while the gut-homing marker α4β7 directly correlated with age (R = 0.4057, p = 0.0085; Table S2) but was not a predictor of age in the GLM analysis (p = 0.978; Figure 1A). This surprising finding suggests that changes in NK cell homing repertoires may be associated with age. We also sought to utilize dimensionality reduction methods to examine the high-dimensional data on a 2-dimensional projection to discern whether the two age groups cluster independently. We first used multidimensional scaling (MDS) and found that both age groups largely overlapped, highlighting that NK cells remain phenotypically consistent during aging (Figure 1B). To further examine the differences between groups of the GLM predictors, we explored mean fluorescence intensity (MFI) expressions for the five strongest log-odds predictors for each group (Figure 1C). These results largely recapitulated the GLM analyses but also indicated that changes during aging most likely involve a multifaceted change in receptor expression.

NK cell aging phenotypes in PWH diverge in people over 50 compared with uninfected controls
Next, we sought to determine the impact of HIV infection on the NK cell aging phenotype seen in HDs. We examined two groups of PWH: those that were either on cART or were not on treatment (viremic) at the time of sampling (Table S1). Using the same GLM with bootstrap resampling as before, we first examined PWH compared with HDs for those under the age of 45. Surprisingly, there were few significant predictors of HIV status in this age group (Figure 2), with only PD-1 and HLA-DR being significant when HDs were compared with those on cART (p = 0.021 and p = 0.021, respectively; Figure 2A). When comparing HDs and viremic under the age of 45, NKG2C and α4β7 were potential predictors of HIV (p = 0.084 and p = 0.084, respectively; Figure 2B) status, while PD-1 was a predictor of the HD group (p = 0.042; Figure 2B). Interestingly, α4β7 had a significant negative correlation with age in both the cART (Table S2) and viremic groups (Table S2), which is inverse to the correlation seen in HD aging (Table S2), again suggesting that aging with HIV causes a shift in NK cell trafficking. In contrast to the under 45 group, many proteins emerged as significant predictors of both HIV groups for the over 50 group. For PWH on cART over the age of 50, CD2, CCR7, and α4β7 emerged as significant predictors compared with HDs (p = 0.014, p = 0.014, and p = 0.031, respectively; Figure 2C). These predictors were consistent in the viremic group (p = 0.021, p = 0.021, p = 0.021, respectively; Figure 2D). In addition, the proteins CCR5, CD85j, CCR7, and HLA-DR were also potential predictors of viremic PWH compared with HDs (p = 0.0945, p = 0.021, p = 0.021, and p = 0.084, respectively; Figure 2D).
To determine if the changes in α4β7 and CCR7 are restricted to NK cells or a general immune change, we examined correlations of α4β7 and CCR7 with age for CD4+ and CD8+ T cells. We found that α4β7 was only significantly correlated with age for CD4+ T cells from the HD group and that CCR7 was only significant for CD4+ T cells from the cART group (Table S3). These data suggested that the trafficking modulation is likely highly specific to NK cells. Additionally, we examined CD56 brightCD16− NK cells and found that CCR7, CXCR6, and CCR5 all correlate with aging in HDs (Table S3). However, α4β7 did not correlate with any group with aging, and only CXCR6 in cART and CCR7 in viremic correlated with age (Table S3). Taken together, these results again strongly indicate that NK cell trafficking is modulated in HIV and aging, as shown by a decreased expression of the gut-homing marker α4β7 and increased expression of the lymph node-homing marker CCR7. Broadly, this change could be considered a reversal of the expected healthy aging homing repertoire of NK cells.

**HIV viral load and duration of known infection predicts NK cell repertoires in PAWH**

NK cell changes in functional capacity, phenotype, exhaustion, and trafficking have been previously shown to correlate with viral load.30,33–36 Due to these previously described correlations, we next aimed to evaluate potential correlates among HIV viral load and observed NK phenotypes in the viremic group, independent of age. Interestingly, viral load did not correlate with NK cell frequency (Table S4), activation/checkpoint markers (Table S4), or NK cell receptors (Table S4). For trafficking markers (Figure 3), α4β7 did show a positive trend with viral...
Figure 2. Analysis of CD56dimCD16+ NK cells in PWH compared to HD

(A–D) GLM with bootstrap resampling was used to determine predictors of (A) HDs (n = 22) and cART (n = 24) under the age of 45; (B) HDs (n = 22) and viremic PWH under the age of 45 (n = 12); (C) HDs (n = 24) and cART (n = 34) over the age of 50; and (D) HDs (n = 24) and viremic PWH (n = 9) over the age of 50. Violin plots show the expression levels of highlighted markers.

(E) Boxplots of MFI expression for all groups and ages for significant markers. Black boxes represent HDs, blue boxes represent cART, and orange boxes represent viremic PWH.

GLM p values are BH adjusted. Boxplot p values were calculated using Kruskal-Wallis test with Dunn’s correction. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Error bars = SEM.

load (R = 0.3758, p = 0.0932; Figure 3A), while only CCR7 significantly correlated with viral load (R = 0.4804, p = 0.0275; Figure 3B). Neither CXCR6 (R = −0.09085, p = 0.5721; Figure 3C) nor CCR5 (R = −0.08389, p = 0.602; Figure 3D) significantly correlated with viral load. To rule out generalized systemic immunological trafficking changes, correlations in CD4+ and CD8+ T cells were performed. We found that CCR7 in CD4+ T cells and CCR5 in CD8+ T cells did correlate with viral load (Table S4), while no correlation was found with the remaining trafficking markers. These analyses reinforce the fact that even independent of aging, HIV infection and ongoing virus replication influences NK cell homing and potential tissue localization.

Given that the duration of HIV infection may play a role in altering NK cell phenotypes within PWH, we performed a correlation analysis between NK cell markers and the duration of known infection for PWH with or without cART (Figure 4; Table S5). Unexpectedly, a significant positive correlation between duration of known infection and frequency of NK cells in PWH on cART was observed (R = 0.3368, p = 0.0097; Table S5). Interestingly, only 2B4 showed a positive trend with duration of infection among viremic PWH (R = 0.3918, p = 0.0790; Table S5). NK-specific receptors did not correlate with duration of infection, except for NKp46 in the cART group, which showed a weak negative correlation (R = −0.2721, p = 0.0388; Table S5), and correlations between duration of known infection and NK trafficking were not observed (Figures 4A–4D). Additionally, no significant trafficking correlations with duration of known infection were observed in either CD4+ or CD8+ T cells.
Collectively, these data suggest that the duration of infection could influence the NK cell repertoire, but rather age and viremia are predictors of the HIV-associated trafficking change.

Elevated CMV IgG titers is linked to higher expression of several NK cell aging phenotypes in those over 50

NK cells are known to undergo phenotypic and functional changes following cytomegalovirus (CMV) infection, mainly through the expansion of an adaptive NK cell pool that is CD57+ and NKG2C+.37–39 To determine if age-related changes observed are due to CMV infection, we evaluated plasma anti-CMV immunoglobulin G (IgG) antibody titers in all cohort groups. Consistent with known data on CMV and HIV co-infections,37 CMV antibody titers were elevated in PWH compared with HDs (Table S1). The CMV + HD group over 50 years of age had higher proportions of NK cells expressing CD57, NKG2C, CD127, PD-1, and CD85j (Table S3), which could represent an expanded adaptive NK subset previously described by our group and others.38–41 Interestingly, we did not see a significant impact of CMV on α4β7 or CCR7 in HDs (Figures 5A and 5B, respectively; Table S6). CXCR6 was also found to not be significantly different between CMV groups in HDs (Figure 5C). However, increased CCR5 expression was observed within CMV titers from both HD age groups (Figure 5D) and positively correlated with age (Table S6). When comparing CMV− and CMV+ samples, regardless of age, we see a lower expression of α4β7 in the CMV+ samples (Figure S4A). With the limited data within range, we do not see a significant correlation in the cART group between CXCR6 expression and CMV IgG titer (R = −0.3721, p = 0.0072; Table S6). However, we do not see the same correlation in the viremic group for CXCR6 (R = 0.3404, p = 0.1311; Table S6). Overall, these states. To help minimize this gap, we used 28-color polychromatic cytometry to robustly characterize cell surface changes in the NK cell repertoire of healthy persons and PAWH both on and off cART. Indeed, we found that an increase in α4β7 expression is one of the tightest predictors of how the global NK cell repertoire changes during healthy aging. Furthermore, we observed a total reversal of this phenomenon in PWH.

Aging has been found to increase the CD56dimCD16+ population of NK cells while decreasing functional capacity and even further contributing to systemic inflammation.24 Our data demonstrate a well-conserved aging NK phenotypic profile consisting of NK cell-specific activation and altered checkpoint-specific receptors. Interestingly, a highly distinctive trafficking pattern was identified with NK cells in aging consisting of decreased lymph node homing via downregulation of CCR7, in conjunction with increased gut mucosae homing via upregulated α4β7. Previous reports have identified age-associated dysfunction in the gut integrity, resulting in microbial translocation and macrophage dysfunction.42 Our data could suggest that increased recruitment of NK cells to the gut mucosae could be one mechanism for maintaining gut homeostasis during aging. Further investigation into the cytolytic capabilities of trafficked NK cells to the gut mucosae can provide more robust insights into the identified trafficking repertoire.

Introduction of cART therapy has dramatically lengthened the lifespan of PWH, resulting in a novel cohort crucial to understanding HIV pathogenesis. One of the most interesting findings in this study was the modulation of NK cell trafficking pattern with HIV infection. Regardless of cART treatment, NK cells from PWH showed a significant decrease in α4β7 expression with a trend toward increased CCR7, potentially indicating a reduction of NK cell trafficking to the gut, but instead mobilizing to the lymph nodes, perhaps related to lymphoid follicles being reservoirs for

Figure 3. Correlation analysis of HIV viral load and protein expression levels on CD56dim CD16+ NK cells in PWH (n = 21)

Trafficking markers analyzed include (A) α4β7, (B) CCR7, (C) CXCR6, and (D) CCR5. Spearman R and p values are noted on each graph.

DISCUSSION

Delineating meaningful changes in NK cell profiles during aging and in PWH has remained poorly understood, limiting a complete understanding of the cellular changes provoked following infection that may result in subsequent secondary disease states. To help minimize this gap, we used 28-color polychromatic cytometry to robustly characterize cell surface changes in the NK cell repertoire of healthy persons and PAWH both on and off cART. Indeed, we found that an increase in α4β7 expression is one of the tightest predictors of how the global NK cell repertoire changes during healthy aging. Furthermore, we observed a total reversal of this phenomenon in PWH.

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(Table S5).
both HIV and SIV. Despite the effectiveness of cART, HIV can survive within follicular helper T cells, remaining undetected by immunoregulatory activation. In non-human primate models, previous reports have compared the follicular regions in chronically infected SIV-pathogenic and non-pathogenic hosts and saw an increase of NK cell recruitment in elite controllers. It is possible that in PWH, NK cells are mobilizing to the lymph nodes in attempt to clear residual HIV-infected T cells. Another interesting finding in our study shows that the change in $\alpha$4$\beta$7 is only observed in older PWH on cART but not PWH off treatment. This decrease in $\alpha$4$\beta$7 was not correlated with viral load as well but did correlate with duration of known infection in PWH on cART. These findings may be indicative that prolonged immunological pressure from HIV could be driving this reversal of NK cell gut homing.

We also compared putative phenotypic indicators for aging NK cells in infected and uninfected individuals under 45 or over 50. Surprisingly, we found that younger persons had NK cell repertoires that are relatively similar regardless of HIV status, but noticeable disparities became clearly apparent in those over the age of 50. CD2, CCR7, and $\alpha$4$\beta$7 were significant predictors compared with HDs for PWH on cART, with the addition of CCR5 and CD85j for untreated PWH. Our data suggest that neither aging nor HIV alone severely impacts the NK cell repertoire but perhaps rather a combination of the two does. Aging and HIV are associated with a variety of similar cellular perturbations relating to function, proliferation, and exhaustion. A comparison of elderly HIV-infected individuals with HDs in the same age cohort found increased rates of hypertension, hypertriglyceridemia, and other disorders in HIV-infected individuals. It is possible that a combination of the two have a synergistic and significant effect on the NK cell phenotypic profile. Altogether, these data highlight the age-associated NK cell phenotypic changes in HIV-infected adults and their potential clinical implications.

In line with previous research, we identified NK cells from CMV-infected aged adults to have higher expression of CD57 and NKG2C, a unique subset of adaptive NK cells primed by the virus. Although we found age-related NK cell phenotypic changes also associated with CMV status, there was minimal impact of CMV infection specifically on NK trafficking receptor expression in HDs. Specifically, there was no significant difference between CMV+ and CMV− aged individuals for NK cell $\alpha$4$\beta$7, CCR7, or CXCR6. These data suggest that age-related changes in the NK cell repertoire do occur due to CMV infection but are largely independent from those induced by HIV.

Importantly, we acknowledge several limitations in our study, which were predominantly focused on limited treatment information and sample quality issues. Due to the age or other limitations of some samples, we were unable to obtain CMV viral shedding from cryopreserved urine samples. In addition, treatment history, specific antiretroviral drugs used, and any potential interruptions in treatment were not known for all participants. Many study participants were on ART regimens that are no longer common care, thus the impact of other drugs on NK cells and chronic inflammation will need to be further examined. Finally, it is also important to acknowledge that despite the large number of participants evaluated, it is difficult to ascertain the full relative contribution of so many factors. This first study provides important indicators on the role of trafficking markers specifically in aging PWH, but subsequent much larger patient studies will be needed in the future.

Altogether, we provide an aging NK cell phenotypic profile, delineate the modulations induced by HIV infection, and highlight the combined dysfunctional properties of HIV and aging. To our knowledge, this is the first study of NK cell phenotypes in aging PWH in both treated and untreated aged cohorts. NK cells have been linked to the control and disease progression of HIV, making it imperative to understand the natural trafficking patterns of these cells and the subsequent modulations following HIV infection. Further research will be needed to evaluate the functional capabilities of aged NK cells in PWH on and off cART and the consequences of these changes.

**Limitations of the study**

While the study described highlights a significant disruption in NK cell homing, particularly to the gut, there are several key...
limitations. First, the study used cryopreserved samples, which could have had some minor impacts on phenotype. Second, some information on specific ART regimens the patients were on at time of collection or treatment history is incomplete and may differ from patient to patient. Finally, while there is a sufficient number of samples per group to draw statistical significance for homing patterns, a larger cohort may be needed to validate some minor marker differences. In addition, all samples were collected from individuals living in Hawai‘i at the time of study, so there may be some population bias.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100773.

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AUTHOR CONTRIBUTIONS

L.C.N. and R.K.R. designed the study. K.W.K., S.V.S., O.A.L., T.A.P., M.J.C., M.M., G.W., and S.B. performed the experiments and analyzed data. K.W.K. performed the bioinformatic analyses. C.M.S. provided samples critical to the study. All authors contributed to the writing of the manuscript.

DECLARATION OF INTERESTS

All authors report no competing interests.
We support inclusive, diverse, and equitable conduct of research.

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56. Seiler C. CytoGLMM Workflow.
# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Blue LIVE/DEAD Amine| Invitrogen | Cat#L23105 |
| CD19-PE-CY5.5       | Beckman Coulter | Clone J3-119; Cat#A66328 |
| CD14-BUV615         | BD Pharmingen | Clone MSE2; Cat#751150; RRID:AB_275174 |
| CD3-BUV496          | BD Pharmingen | Clone UCHT1; Cat#564809; RRID:AB_2744388 |
| CD4-BB700           | BD Pharmingen | Clone L200; Cat#566479; RRID:AB_2739738 |
| CD56-BUV737         | BD Pharmingen | Clone NCA16.2; Cat#612766; RRID:AB_2813880 |
| CD16-BUV563         | BD Pharmingen | Clone 3G8; Cat#748851; RRID:AB_2873254 |
| NKP46-BV711         | BD Pharmingen | Clone 9E2 /NKP46; Cat#563043; RRID:AB_2737971 |
| NKG2D-BB790         | BD Pharmingen | Clone 1D11; Custom Conjugate |
| NKG2C-Biotin        | MILTENYI | Clone REA205; Cat#130-120-448; RRID:AB_2801764 |
| Streptadivin-BUV395 | BD Pharmingen | Cat#564176; RRID:AB_2869553 |
| NKGA-PE-CY7         | BECKMAN COULTER | Clone Z199; Cat#B10246; RRID:AB_2687887 |
| KIR3DL1/S1-VioBlue  | MILTENYI | Clone REA168; Cat#130-110-447; RRID:AB_2655352 |
| KIR2DL1/S1/S3/S5-FITC| BIOLEGEND | Clone HP-MA4; Cat#339504; RRID:AB_2130378 |
| KIR2DS4-APC-Vio770  | MILTENYI | Clone REA284; Cat#130-114-618; RRID:AB_2655374 |
| CD8βj-PE            | BD Pharmingen | Clone GHI/75; Cat#551053; RRID:AB_394020 |
| a4b7-APC            | NHP Reagent Resource | Clone A4B7R1; RRID:AB_2819257 |
| CXCR6-BV786         | BD Pharmingen | Clone 13B15E; Cat#743602; RRID:AB_2741614 |
| CCR5-PE-CY5         | BD Pharmingen | Clone 2D7/CCR5; Cat#566899; RRID:AB_396529 |
| CCR7-APC-R700       | BD Pharmingen | Clone 3D12; Cat#565876; RRID:AB_2744304 |
| 2B4-BV650           | BD Pharmingen | Clone 2–69; Custom Conjugate |
| CD2-BV510           | BIOLEGEND | Clone RPA2.10; Cat#300218; RRID:AB_2566040 |
| CD8α-BV570          | BIOLEGEND | Clone RPA-78; Cat#301038; RRID:AB_2563213 |
| CD57-BB630          | BD Pharmingen | Clone HNK-1; Custom Conjugate |
| KLRG1-PE-Dazzle594  | BIOLEGEND | Clone 2F1/KLRG1; Cat#138424; RRID:AB_2564051 |
| PD1-BV605           | BD Pharmingen | Clone EH12.1; Cat#563245; RRID:AB_2738091 |
| TIM-3-BV750         | BD Pharmingen | Clone 7D3; Custom Conjugate |
| HLA-DR-BUV661       | BD Pharmingen | Clone G46-6; Cat#612980; RRID:AB_2870252 |
| IL-7R-BUV805        | BD Pharmingen | Clone HIL-7R-M21; Cat#748486; RRID:AB_2872901 |
| **Biological samples** | | |
| Human Peripheral Blood Mononuclear Cells | University of Hawai‘i | |
| Human Plasma | University of Hawai‘i | |
| **Critical commercial assay** | | |
| Cytomegalovirus IgG and IgM antibody titers | Quest Diagnostics | Cat#6732 |
| Amplicor HIV-1 Monitor Ultrasensitive Assay | Roche | |
| **Deposited data** | | |
| Analyzed data | This paper | N/A |
| Raw flow cytometry data | | |
| **Software and algorithms** | | |
| FlowJo v10.7.1 | BD Biosciences | https://www.flowjo.com/ |
| Prism v9.0 | GraphPad | https://www.graphpad.com/ |
| R v3.6.3 | R Foundation | https://www.r-project.org/ |
| RStudio | RStudio | https://www.rstudio.com/ |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, R. Keith Reeves, Ph.D. (keith.reeves@duke.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cryopreserved human peripheral blood mononuclear cells (PBMCs) were obtained from the Hawai‘i Aging with HIV-1 Cohort (HAHC) at University of Hawai‘i. Details of the HAHC study enrollment and clinical characterization were previously published and approved by the University of Hawai‘i Institutional Review Board. All participants signed institutional review board–approved informed consent forms prior to participation. CD4 lymphocyte counts were obtained in real-time by standard technique from a local CAP certified reference laboratory. Demographic and clinical details of study participants are provided in Table S1.

METHOD DETAILS

Flow cytometry
Flow cytometry staining of PBMCs was performed using a 28-color surface phenotype panel (key resources table). Briefly, cryopreserved PBMCs were rapidly thawed at 37 °C and immediately transferred to complete media pre-warmed to 37 °C. After centrifugation, PBMCs were incubated for 30 min at 4 °C in Blue Live/Dead (Invitrogen, Carlsbad, CA, USA). PBMCs were then washed, and surface stained for 20 min at room temperature (RT). Samples were then stained immediately after with Streptavidin-BUV395 as a secondary antibody for biotinylated CD159c for 15 min at RT. PBMCs were washed once more and fixed with 2% paraformaldehyde. All acquisitions were carried out on a FACS Symphony cytometer (BD Biosciences) and analyzed with FlowJo v10.7.1 (BD Biosciences). The gating strategy used to define NK cells was as follows: Live, Lineage− (CD19− CD14− CD4− CD3−), CD56dimCD16+. Median fluorescent intensity (MFI) values were exported from FlowJo. To minimize the potential for batch effects, we took several steps to mitigate these during the study planning. First, we ordered antibody in bulk to ensure that single lots were used for the entire duration of the study. Second, the BD FACS Symphony underwent daily quality control to ensure that the lasers, filters, and voltages were within acceptable and consistent values. Finally, we were blinded to the status of the samples during data acquisition which resulted in adequate group mixing for each analysis day.

CMV antibody titer quantification
Matched cryopreserved plasma samples were used for quantification of Human Cytomegalovirus IgG and IgM antibody titers (Quest Diagnostics; order code 6732; Marlborough, MA, USA).

HIV viral load quantification
Plasma viral loads were assessed using Amplicor HIV-1 Monitor Ultrasensitive Assay (Roche Molecular System, Branchburg, NJ, USA).

QUANTIFICATION AND STATISTICAL ANALYSIS

Spearman correlation analysis was performed in Prism v9.0 (GraphPad Software, San Diego, California, USA). Generalized linear modeling (GLM) with bootstrapping was performed with the CytoGLMM R package with R v3.6.3.52 GLM with bootstrapping was performed according to previously published studies. Briefly, compensated FCS files of CD56dimCD16+ NK cells were exported from FlowJo and loaded into R. Data were transformed using the hyperbolic sine transformation with a cofactor equal to 5 and a random sampling of 2,000 cells from each sample, or all cells if the sample contained fewer than 2,000 cells, was performed. The cytoglm function of the CytoGLMM package was used to perform 1,000 iterations of bootstrap resampling with replacement followed by logistic regression. Results are reported as log-odds of a given marker predicting binary group assignment (HD and ART; HD and PWH; HD under 45 and HD over 50). Corrections for multiple testing were performed using the Benjamini-Hochberg (BH) method for controlling false discovery rate. P-values reported are BH adjusted p-values. An adjusted p-value cutoff of 0.05 was used to
determine significance. Median fluorescence intensities (MFI) were exported from FlowJo for comparing marker expression between groups. These data were not transformed prior to statistical analysis. Mann-Whitney U-tests were used to compare MFI levels between groups.

**Multidimensional analyses**
Multidimensional scaling (MDS) was performed using the CytoGLMM R package as described by package documentation using median summarized expression values for each sample.