Elevated levels of invariant natural killer T-cell and natural killer cell activation correlate with disease progression in HIV-1 and HIV-2 infections

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Objective: In this study, we aimed to investigate the frequency and activation of invariant natural killer T (iNKT) cells and natural killer (NK) cells among HIV-1, HIV-2, or dually HIV-1/HIV-2 (HIV-D)-infected individuals, in relation to markers of disease progression.

Design: Whole blood samples were collected from treatment-naive HIV-1 (n = 23), HIV-2 (n = 34), and HIV-D (n = 11) infected individuals, as well as HIV-seronegative controls (n = 25), belonging to an occupational cohort in Guinea-Bissau.

Methods: Frequencies and activation levels of iNKT and NK cell subsets were analysed using multicolour flow cytometry, and results were related to HIV-status, CD4\textsuperscript{+} T-cell levels, viral load, and T-cell activation.

Results: HIV-1, HIV-D, and viremic HIV-2 individuals had lower numbers of CD4\textsuperscript{+} iNKT cells in circulation compared with seronegative controls. Numbers of CD56\textsuperscript{bright} NK cells were also reduced in HIV-infected individuals as compared with control study participants. Notably, iNKT cell and NK cell activation levels, assessed by CD38 expression, were increased in HIV-1 and HIV-2 single, as well as dual, infections. HIV-2 viremia was associated with elevated activation levels in CD4\textsuperscript{+} iNKT cells, CD56\textsuperscript{bright}, and CD56\textsuperscript{dim} NK cells, as compared with aviremic HIV-2 infection. Additionally, disease markers such as CD4\textsuperscript{+} T-cell percentages, viral load, and CD4\textsuperscript{+} T-cell activation were associated with CD38 expression levels of both iNKT and NK cells, which activation levels also correlated with each other.

Conclusion: Our data indicate that elevated levels of iNKT-cell and NK-cell activation are associated with viremia and disease progression markers in both HIV-1 and HIV-2 infections.

Keywords: CD38, HIV-1, HIV-2, immune activation, invariant natural killer T cells, natural killer cells, viremia

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Introduction

HIV type 1 (HIV-1) and type 2 (HIV-2) are related viruses that can cause AIDS. Although HIV-1 has given rise to a pandemic, HIV-2 is mainly restricted to West Africa. In contrast to HIV-1, long-term nonprogressors are common in HIV-2 infection [1]. The reason for this is not fully understood, but HIV-2 infection is characterized by a large proportion of infected individuals with low or undetectable plasma viral load, and a viral load set point at least one log lower than in HIV-1 infection [2,3]. However, HIV-2-infected individuals with detectable viremia progress faster to AIDS and have higher mortality than HIV-2-infected aviremic study participants [4–6].

The innate and adaptive immune responses to HIV-1 have been studied extensively, whereas much less is known about immune responses in HIV-2 and HIV-1/2 dual (HIV-D) infections. Nevertheless, it has been reported that the breadth and potency of virus specific T-cell responses, as well as neutralizing antibodies, distinguish HIV-2 from HIV-1 [7–11]. Additionally, HIV-2 has been reported to delay subsequent HIV-1 disease progression in HIV-D-infected individuals [12,13]. Furthermore, HIV-2-infected individuals, compared with HIV-1, display lower levels of chronic T-cell immune activation [14]. Still, T-cell activation in HIV-2 infection appears to be related to CD4+ T-cell levels as well as viral load, similar to HIV-1 infection [15–17].

HIV-1 disease progression is associated with chronic immune activation, characterized by elevated frequencies of T cells, expressing activation markers, such as CD38 and HLA-DR [18] (and reviewed in [19,20]). Still, the cause or consequence relationship between virus replication and chronic immune activation has not been fully elucidated. Chronic immune activation is also associated with augmented levels of bystander activating factors such as proinflammatory cytokines, markers of inflammation, and microbial translocation which are detected in progressive infection of both HIV-1 and HIV-2 [21–24]. Many of these bystander factors are produced by activated innate immune cells, which include monocytes, macrophages, and dendritic cells [19,20]. Increased activation of natural killer (NK) cells occurs in chronic HIV-1 infections [25–27], and such activation can be associated with dysfunctional NK cell responses [28].

Innate lymphocytes such as NK and invariant natural killer T (iNKT) cells play an important role in the host defence against viruses including HIV [29,30]. Dramatic shifts in the population dynamics of NK cells are observed during chronic HIV infection, for example the expansion of the CD56dim NK cell population [31]. The function of NK cells has been shown to be less impaired during HIV-2 compared with HIV-1 infection [32]. iNKT cells have characteristics of both NK and T cells and rapidly produce high levels of proinflammatory cytokines such as IFNγ and tumour necrosis factor [33] (and reviewed in [34]). About one-third of the iNKT population expresses CD4 and can be infected by HIV-1 [35–37]. Furthermore, during untreated HIV-1 infection, iNKT cells are known to decline in numbers and develop an exhausted, dysfunctional phenotype [36–39].

Comparative studies on the phenotypes of iNKT and NK cells during HIV-1, HIV-2, and HIV-D infections, and the relation to viremia and CD4+ T-cell suppression, are lacking. In this cross-sectional study, we investigated the frequency and phenotype of NK and iNKT cells in individuals infected with, but not treated for, these viruses. Results revealed that iNKT and NK cell activation correlates with disease severity, as indicated by levels of CD4+ T cells, viral load, and CD4+ T-cell activation, in both HIV-1 and HIV-2 infections.

Materials and methods

Study population

The individuals included in this study were part of an occupational cohort of police officers in Guinea-Bissau [40,41] (Table 1 and Supplementary Methods in Supplemental Digital Content, http://links.lww.com/QAD/A929). Blood samples were from treatment-naive HIV-1 (n = 23), HIV-2 (n = 34), or HIV-D (n = 11) infected individuals, as well as from 25 HIV-seronegative individuals from the same cohort. The study was approved by the ethical committee in Guinea-Bissau and by the ethical committee at Lund University.

Blood sampling, HIV status, and CD4+ T-cell determination

Venous blood samples were drawn and collected in vacutainer tubes (BD Biosciences, San Jose, California, USA) with EDTA as anticoagulant and in Cyto-Chex BCT tubes (Streck, Omaha, Nebraska, USA). Plasma obtained from EDTA tubes was kept frozen at −80°C until use. HIV infection status was determined by serology, and the percentage of CD4+ T cells was determined on fresh whole blood by flow cytometry, as described [42]. The rational for choosing %CD4+ T cells as a marker of disease progression in the HIV-infected cohort was based on findings suggesting that %CD4+ T cells is a more stable disease marker than absolute CD4+ T-cell count in settings with elevated pathogenic burden and comorbidities [43] and on the prior use of this marker in the studied cohort [44]. Determination of absolute number of CD4+ T cells/μL (CD4+ T-cell count) was performed at the Laboratory of Clinical Immunology and Transfusion Medicine, Skåne University Hospital, Lund, Sweden on Cyto-Chex stabilized whole blood.
Table 1. Cohort characteristics.

| Parameters                  | HIV-1   | HIV-2   | HIV-D   | Controls |
|-----------------------------|---------|---------|---------|----------|
| Numbers (women/men)         | 23 (10/13) | 34 (8/26) | 11 (2/9) | 25 (10/15) |
| Age (years)                 | 46 (41–53) | 53 (48–58) | 50 (44–52) | 48 (40–55) |
| % CD4<sup>+</sup> T cells   | 12.3 (6.4–25.6) | 25.4 (17.9–35.9) | 15.3 (11.6–21.6) | 42.3 (38–50) |
| Viral load (RNA copies/ml)* | 12 009 (2799–30067) | <75 (<75–134) | 2230 (1147–6398) | NA |

*Median (IQR).

NA, not applicable.

**Plasma viral load**

With minor modifications, plasma HIV-1 and HIV-2 viral loads were determined by in-house quantitative PCR protocols, as described previously [45]. In brief, viral RNA was extracted and purified using the miRNeasy micro Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany), and TaqMan qRT-PCR was performed using the Superscript III Platinum One Step qRT-PCR kit (Life Technologies, Carlsbad, California, USA). The detection limits for the HIV-1 and HIV-2 quantifications were 75 RNA copies/ml in plasma from HIV-1 or HIV-2 singly infected and 135 RNA copies/ml in plasma from HIV-D-infected.

**Immunophenotyping**

Immunophenotyping of lymphocytes was performed on whole blood collected and stabilized in Cyto-Chex BCT tubes (7–11 days) before multicolour flow cytometry [46]. Before antibody labelling, red blood cells were lysed using 1× lysis buffer (BD FACS) and washed with PBS containing 2 mmol/l EDTA. The panel for the antibody staining is shown in Table S1, Supplemental Digital Content, http://links.lww.com/QAD/A929. Multicolour flow cytometry was performed using a BD LSORTeza, and data were analysed using FlowJo software Version 9.6.2 (LLC, Ashland, Oregon, USA) (for details see Supplementary Methods and Fig. S1, in Supplemental Digital Content, http://links.lww.com/QAD/A929). Prior to the analysis of whole blood samples from the study participants, the selected panel of antibodies was tested on whole blood collected in EDTA vacutainer tubes, stained and analysed the same day; and stabilized and stored in Cyto-Chex tubes from corresponding donors before staining and analysis. Results, as depicted in Supplemental Fig. S2, http://links.lww.com/QAD/A929, showed that the gated and analysed iNKT and NK cell subsets in the Cyto-Chex stabilized blood were distinct and similar to that of the freshly analysed blood. The absolute numbers of iNKT cells and NK cells/μl were calculated according to %iNKT cells (or NK cells)/100 × CD4<sup>+</sup> T-cell count/%CD4<sup>+</sup> T cells/100. Mean fluorescence intensities from cell subsets with 25 or more events were included in the analysis of CD38 and transcription factor (TF) expression.

**Statistical analysis**

Nonparametric statistical methods for comparison between groups included the Kruskal–Wallis test, with Dunn’s post-test, and the Mann–Whitney U test. Correlations were done with the nonparametric Spearman’s rank-correlation test, and the analyses were performed using the GraphPad Prism 6.0 software (GraphPad Software, San Diego, California, USA). Multiparameter analysis using univariate general linear modelling was performed with SPSS version 22 (IBM Analytics, Armonk, New York, USA).

**Results**

**Characteristics of study participants**

Blood samples were obtained cross-sectionally from treatment-naive HIV-1 (n = 23), HIV-2 (n = 34), or HIV-D (n = 11) infected study participants and HIV-seronegative controls (n = 25) belonging to an occupational cohort residing in Guinea-Bissau (Table 1 and [40,41]). The median ages of the study groups ranged between 46 and 53 years, with HIV-1-infected study participants being 7 years younger than the HIV-2-infected participants (P < 0.05). With regard to the immunological status, the median level of % CD4<sup>+</sup> T cells was for all HIV-infected groups lower than for the uninfected group (P < 0.001). HIV-1, compared with HIV-2, infected individuals also had lower %CD4<sup>+</sup> T cells (median 12.3 vs 25.4; P < 0.01). Furthermore, all HIV-1-infected, singly or dually, had higher median viral loads than the HIV-2-infected participants (median 12 009, 2230 vs <75 RNA copies/ml; P < 0.001 and P < 0.01, respectively). Indeed, many (74%) of the HIV-2-infected individuals had undetectable plasma viral loads and were therefore classified as aviremic.

**Invariant natural killer T and natural killer cell subsets are reduced in HIV-infected individuals**

iNKT and NK cell numbers in peripheral blood are often reduced in HIV-1-infected individuals [36,37,39,47,48]. In contrast, studies investigating innate lymphocytes in HIV-2 are limited [1]. Here we compared the frequency of total and CD4<sup>+</sup> iNKT cells as well as CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells in whole blood of HIV-seronegative controls, HIV-1, HIV-2, and HIV-D-infected individuals (Fig. 1a and b). Absolute numbers of both total iNKT and CD4<sup>+</sup> iNKT cell subsets of HIV-1 and HIV-D-infected study participants were, or tended to be, decreased compared with controls (P < 0.001 to P = 0.09). Moreover, all HIV-infected groups had also reduced
numbers of CD56\textsuperscript{bright} NK cells compared with the controls (\(P<0.01\) to \(P<0.05\)), whereas the impact on the CD56\textsuperscript{dim} NK cell numbers was less pronounced. The proportion of iNKT and NK cell subsets within the lymphocyte population was also less affected (Fig. S3 in Supplemental Digital Content, http://links.lww.com/QAD/A929).

Viremia is infrequently detected in HIV-2-infected individuals, but once occurring, it is linked to disease progression similar to that seen in HIV-1 infection [4,5,49]. By comparing HIV-2 singly infected study participants, being either viremic or aviremic (Fig. 1c), we found that the absolute numbers of CD4\textsuperscript{+} iNKT cells were reduced in viremic HIV-2 infection (\(P<0.05\)), and likewise the CD56\textsuperscript{dim} NK cells tended to be fewer (\(P=0.07\)). The percentage of CD56\textsuperscript{dim} NK cells was lower in HIV-2 viremic study participants, and a similar trend was noted for the CD4\textsuperscript{+} iNKT cells (\(P<0.05\) and \(P=0.05\), respectively, Fig. 1c in Supplemental Digital Content, http://links.lww.com/QAD/A929). However, there was no difference in the percentage or absolute numbers of total iNKT cells, nor CD56\textsuperscript{bright} NK cells (Fig. 1c and Fig. S3 in Supplemental Digital Content, http://links.lww.com/QAD/A929). Reduced numbers of CD56\textsuperscript{bright} NK cells were instead observed in the aviremic HIV-2 study participants compared with the HIV-seronegative controls (\(P<0.01\); data not shown).

Natural killer and invariant natural killer T-cell subsets display enhanced activation in HIV infections

In both HIV-1 and HIV-2 infections, excessive T-cell activation is correlated with the progression to AIDS [16,17]. However, less is known about the link between elevated activation of NK cells and iNKT cells and disease progression.

Fig. 1. Invariant natural killer T and natural killer cell subsets are reduced in HIV-infected individuals. Absolute numbers of invariant natural killer T (iNKT) and natural killer (NK) cell subsets assessed in blood samples of HIV-1, HIV-2, HIV-D, and seronegative control individuals. (a) The gating strategy for the different iNKT and NK cell subsets. (b) The total invariant natural killer T-cell (filled squares), CD4\textsuperscript{+} iNKT cell (open squares) CD56\textsuperscript{dim} NK cell (open circles), and CD56\textsuperscript{bright} NK cell (filled circles) absolute numbers per \(\mu\)l of blood. Comparisons of absolute numbers of (c) total iNKT, CD4\textsuperscript{+} iNKT, CD56\textsuperscript{dim} NK cell and CD56\textsuperscript{bright} NK cell subsets (labelled as described above) in HIV-2 aviremic (viral load \(\leq 75\) RNA copies/ml blood) and viremic individuals (viral load \(>75\) RNA copies/ml). The dashed line represents the median absolute number of respective cell type in HIV-seronegative individuals. Median is depicted as solid bars. The Kruskal–Wallis test with Dunn’s post test, and the Mann–Whitney test were used where appropriate. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).
progression. Thus, we set out to analyse the level of CD38 expression on these cells in HIV-1, HIV-2, and HIV-D-infected individuals as well as HIV-seronegative controls (Fig. 2a). CD38 expression levels on total iNKT, CD4⁺ iNKT, and CD56bright NK cells were significantly elevated in both HIV-1 and HIV-2 singly infected study participants (P < 0.0001 to P < 0.05) compared with the controls. HIV-D-infected individuals also had increased CD38 expression on iNKT cells and CD56bright NK cells (P < 0.01 and P < 0.05, respectively). Furthermore, the activation levels of both CD56dim and CD56bright NK cells were higher in HIV-1 compared with HIV-2-infected individuals (P < 0.01).

As T-cell activation has been shown to correlate with viral load in both HIV-1 and HIV-2 infections [15], we next compared CD38 expression levels on iNKT cells and NK cells in patients with viremic and aviremic HIV-2 infection (Fig. 2b). Elevated levels of CD38 expression on CD4⁺ iNKT, CD56dim as well as CD56bright NK cells distinguished the viremic state of HIV-2 infection (P < 0.05). Furthermore, CD38 expression on total iNKT, CD4⁺ iNKT, and CD56bright NK cells of aviremic HIV-2-infected study participants were elevated compared with controls (P < 0.01; Fig S4 in Supplemental Digital Content, http://links.lww.com/QAD/A929). Taken together, these findings suggest that elevated activation of iNKT and NK cell subsets is a shared feature between HIV-1 and HIV-2 infections.

Altered immune activation of T lymphocytes in HIV infections is potentially influenced by the expression of certain TFs [50]. Promyelocytic leukaemia zinc finger (PLZF) and T-bet have been implicated in the development and activation of iNKT cells and NK cells [51–55]. Here, no difference in relative PLZF or T-bet levels among iNKT cells was observed between the groups (Fig. S5 in Supplemental Digital Content, http://links.lww.com/QAD/A929). We noted instead that relative PLZF levels in CD56dim NK cells were reduced in HIV-D-infected individuals compared with the controls (P < 0.001), with a trend towards reduced levels in the single HIV-1 and HIV-2 infections. In the CD56bright NK cell subset, relative levels of T-bet were elevated in HIV-1 infection compared with controls (P < 0.05).

**Invariant natural killer T and natural killer cell activation in HIV infection correlate with CD4⁺ T cell level and viral load**

We next analysed if iNKT cell and NK cell activation levels correlated with viral load or %CD4⁺ T cells among the HIV-infected individuals. CD38 levels on both total

**Fig. 2. Invariant natural killer T and natural killer cells display enhanced activation in HIV infections.** Degree of activation represented by the median fluorescence intensity (MFI) level of CD38 surface expression was assessed in blood samples of HIV-1, HIV-2, HIV-D-infected individuals and controls. (a) Comparison of CD38 MFI expression levels on total invariant natural killer T (iNKT; filled squares), CD4⁺ iNKT (open squares), CD56dim (open circles), and CD56bright natural killer cells (NK; filled circles) between HIV-infected groups and seronegative controls. (b) CD38 MFI levels on iNKT and NK cell subsets in aviremic and viremic HIV-2+ individuals (as defined in Fig. 1c). The dashed line represents the median CD38 MFI level of noninfected individuals. Median depicted as solid bars for the different groups. Kruskal–Wallis test with Dunn’s post test was used for multiple comparisons, whereas the Mann–Whitney U test was used for comparison of two groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
iNKT cells and CD4\(^+\) iNKT cells (Fig. 3a) correlated directly with viral load \((r = 0.33, P < 0.01\) and \(r = 0.48, P < 0.001\), respectively), and inversely with CD4\(^+\) T cells \((r = -0.36, P < 0.01\) and \(r = -0.36, P < 0.05\), respectively). Furthermore, strong correlations were observed between levels of CD38 on CD56\(^{dim}\) and CD56\(^{bright}\) NK cells (Fig. 3b) and viral load \((r = 0.52, P < 0.0001\) and \(r = 0.65, P < 0.0001\), respectively) and %CD4\(^+\) T cells \((r = -0.41, P < 0.001\) and \(r = -0.57, P < 0.0001\), respectively). There were no significant correlations between the iNKT and NK cell expression levels of TFs and CD38 (data not shown).

To further delineate the modulators of CD38 expression, we performed analyses using univariate general linear modelling, including HIV status, age, sex, log%CD4\(^+\) T cells, and log viral load. These analyses supported the conclusion that %CD4\(^+\) T cells, independently of HIV infection type, associated with activation levels assessed by CD38 expression on CD56\(^{dim}\) and CD56\(^{bright}\) NK cells, as well as total iNKT cells (Table S2 in Supplemental Digital Content, http://links.lww.com/QAD/A929). Furthermore, the CD4\(^+\) iNKT cell activation levels were associated with viral load.

**Coordinated elevation of immune activation in innate and adaptive cell subsets**

Little is known about the correlations between levels of activation of innate and adaptive lymphocyte subsets in HIV infections. We therefore analysed the relationships between CD38 expression levels on CD4\(^+\) T cells and NK as well as iNKT cell subsets. Our data revealed relatively strong correlations between activation levels of CD4\(^+\) T cells (Fig. 4a) and those of total iNKT cells \((r = 0.55, P < 0.0001)\), CD4\(^+\) iNKT cells \((r = 0.79, P < 0.0001)\), CD56\(^{dim}\) NK cells \((r = 0.67, P < 0.0001)\), and CD56\(^{bright}\) NK cells \((r = 0.67, P < 0.0001)\). We also noticed that activation levels of the total iNKT and the CD4\(^+\) iNKT cell subsets positively correlated with both NK cell subpopulations (Fig. 4b), and most significantly with CD4\(^+\) T cells \((r = 0.76, P < 0.0001)\). Thus, these findings suggest that elevated activation levels can be detected concomitantly in different lymphocyte populations, representing both innate and adaptive cells.

**Discussion**

Here we investigated frequencies and activation levels of iNKT cells and NK cells in treatment-naive HIV-1 and HIV-2 singly-infected or dually-infected individuals, as well as HIV-seronegative controls. Our results indicate that chronic infection of both HIV-1 and HIV-2 type leads to elevated activation of both iNKT cells and NK cells.

The phenotype of iNKT cells in chronic HIV-1 infection is characterized by elevated expression of the PD-1 exhaustion marker, and an impaired ability to produce IFN-γ [38]. In this study, we found that individuals infected with different types of HIV had iNKT cells with elevated CD38 expression, reflecting an increased activation status. Our results indicated also that viremic HIV-2+ individuals have CD4\(^+\) iNKT cells with increased levels of CD38, as compared with the study participants with aviremic HIV-2 infection. It should,
however, be noted that the number of study participants with viremic HIV-2 infection was limited and firm conclusions are therefore difficult to draw. Still, a similar pattern has been described for conventional T cells in viremic HIV-2-infected individuals displaying T-cell activation comparable with that of HIV-1 infections [16]. Nevertheless, the aviremic HIV-2 study participants, with low or no viremia, had CD4⁺ iNKT cells with CD38 expression levels that were higher than that of the HIV-seronegative controls, which concurs with earlier observations on increased T-cell activation in aviremic HIV-2 infected individuals [17] and HIV-1 elite controllers [56].

In agreement with previous studies describing an association between NK cell activation and HIV-1 disease progression [27,57], we observed that activation of both CD56⁰ and CD56⁺ NK cells was increased in HIV-1 singly infected individuals compared with the controls, but also to the HIV-2-infected individuals. NK cell activation levels in HIV-2 infected study participants with detectable viremia were comparable with those observed in HIV-1. Nevertheless, similar to CD4⁺ iNKT cells, the activation level of CD56⁺ NK cells of aviremic HIV-2 study participants were somewhat elevated compared with the HIV-seronegative controls. This finding stands in contrast to the lack of NK cell activation in HIV-1 elite and viremic controllers reported by others [27]. However, as the majority of the HIV-2 aviremic study participants had been infected for several decades, it is possible that prolonged HIV-2 infection may influence the activation state of the NK cells even in the absence of overt viremia. It is also clear that aviremic HIV-2-infected study participants can have elevated T-cell activation along with declining CD4⁺ T-cell levels [17]. In line with this finding, our univariate general linear modelling analyses revealed that reduced CD4⁺ T-cell level strongly influenced elevated activation of NK cells in an HIV type-independent manner. Analogous impact of CD4⁺ T-cell levels on frequencies of CD38 expressing NK cells was recently reported for HIV-1 infection [27].

Here we observed that activation levels of iNKT and NK cell subsets are highly correlated and that associations exist between the activation levels of these innate lymphocytes and CD4⁺ T-cell activation. This may suggest that iNKT and NK cells contribute to the systemic immune activation, or that this phenomenon is the consequence of such activation. The relationships and kinetics between different factors contributing to chronic immune activation in HIV infections are still not fully elucidated (reviewed in [58]), and may require longitudinal studies from the time of the primary infection, which, especially in the setting of HIV-2 infection, are challenging. Progressive disease in HIV-2 infection has been associated with reduced capacity of NK cells to degranulate, along with altered chemokine and cytokine secretion by CD56⁺ NK cells [32], implying that although NK cell activation is elevated, these cells are not fully functional. Previously, we reported on increased levels of IL-12 in whole blood of HIV-1 and/or HIV-2-infected individuals with progressive disease, indicating that the overall cytokine milieu may activate NK cells [59].

The pattern with loss of CD4⁺ iNKT cells in HIV-1 and dually-infected participants observed here is consistent with previous findings in HIV-1 single infections.
Among the HIV-2-infected individuals, it was mainly those with viremia who had a decrease in CD4⁺ iNKT cells, similar to that seen in the HIV-1-infected participants. Although the CD4⁺ iNKT cell subset can be infected with HIV-1 [60], this has not yet been investigated for HIV-2. Although, not as stark as previously reported [61], we observed either trends or significant reductions in the numbers of CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cell subpopulations during HIV-1 infection. This reduction appeared to be independent of the HIV type. Furthermore, HIV-2 viremia tended to lead to a reduction in CD56<sup>dim</sup> NK cells, whereas the CD56<sup>bright</sup> population did not change in numbers or proportion. This implies that viremia in HIV-2 infection may alter NK cell maturation, a phenomenon also observed in IFNα treatment of chronic hepatitis [62].

HIV-associated immune activation has been related to alterations of transcriptional regulation in CD8⁺ T cells [63,64]. The TFs PLZF and T-bet are important for iNKT and NK cell development, maturation, and function [65–69]; however, their expression in human iNKT cells and NK cells from HIV-infected individuals remains unexplored. We noticed here decreased PLZF levels in the CD56<sup>dim</sup> NK cells of HIV-D-infected individuals, and a downward trend in the CD56<sup>bright</sup> NK cell subpopulation. Furthermore, increased T-bet levels were detected in CD56<sup>bright</sup> NK cells during HIV-1 infection. Additionally, we analysed relationships between the iNKT and NK cell expression levels of TFs and CD38, but found no significant correlations (data not shown). These results should spur further investigations on TFs and their role in functional and developmental modifications of iNKT and NK cells during different types and stages of HIV infections.

In summary, our findings reveal that activation levels of iNKT cells and NK cells are elevated and coordinated in untreated HIV-1, HIV-2, and HIV-D infections. Additionally, we show that elevated activation of both iNKT and NK cells is associated with reduced CD4⁺ T-cell percentages and higher viral loads, in a largely HIV type-independent manner.

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Conflicts of interest

There are no conflicts of interest.

References

1. Nyamweya S, Hagedus A, Jaye A, Rowland-Jones S, Flanagan KL, Macallan DC. Comparing HIV-1 and HIV-2 infection: Lessons for viral immunopathogenesis. Rev Med Virol 2013; 23:221–240.
2. Berry N, Ariyoshi K, Jaffar S, Sabally S, Corrah T, Tedder R, et al. Low peripheral blood viral HIV-2 RNA in individuals with high CD4 percentage differentiates HIV-2 from HIV-1 infection. J Virol 1998; 72:457–468.
3. Andersson S, Norrgren H, da Silva Z, Biague A, Bamba S, Kwok S, et al. Plasma viral load in HIV-1 and HIV-2 singly and dually infected individuals in Guinea-Bissau, West Africa: significantly lower plasma virus set point in HIV-2 infection than in HIV-1 infection. Arch Intern Med 2000; 160:3286–3293.
4. Ariyoshi K, Jaffar S, Alabi AS, Berry N, Schim van der Loeff M, Sabally S, et al. Plasma RNA viral load predicts the rate of CD4 T cell decline and death in HIV-2-infected patients in West Africa. AIDS 2000; 14:339–344.
5. Hansmann A, Schim van der Loeff MF, Kaye S, Awasana AA, Sarge-Njie R, O’Donovan D, et al. Baseline plasma viral load and CD4 cell percentage predict survival in HIV-1- and HIV-2-infected women in a community-based cohort in The Gambia. J Acquir Immune Defic Syndr 2005; 38:335–341.
6. van der Loeff MF, Larke N, Kaye S, Berry N, Ariyoshi K, Alabi A, et al. Undetectable plasma viral load predicts normal survival in HIV-2-infected people in a West African village. Retrovirology 2010; 7:46.
7. de Silva TI, Aasa-Chapman M, Cotten M, Hue S, Robinson J, Bibollet-Ruche F, et al. Potent autologous and heterologous neutralizing antibody responses occur in HIV-2 infection across a broad range of infection outcomes. J Virol 2012; 86:930–946.
8. Duvall MG, Precopio ML, Ambrozak DA, Jaye A, McMichael AJ, Whittle HC, et al. Polynontional T cell responses are a hallmark of HIV-2 infection. Eur J Immunol 2008; 38:350–363.
9. Kong R, Li H, Bibollet-Ruche F, Deckter JM, Zheng NN, Gottlieb GS, et al. Broad and potent neutralizing antibody responses elicited in natural HIV-2 infection. J Virol 2012; 86:947–960.
10. Leighgowitch A, Vindan LM, Omyango C, Sarge-Njie R, Alabi A, Cotten M, et al. Robust Gag-specific T cell responses characterize viremia control in HIV-2 infection. J Clin Invest 2007; 117:3067–3074.
11. Özkaya Sahin G, Holmgren B, da Silva Z, Nielsen J, Nowroozalizadeh S, Esbjörnsson I, et al. Potent intratypic neutralizing activity distinguishes human immunodeficiency virus type 2 (HIV-2) from HIV-1. J Virol 2012; 86:961–971.
12. Ebsjornsson J, Mansson F, Kvist A, Isberg PE, Biague AJ, da Silva ZI, et al. Increased survival among HIV-1 and HIV-2 dual-infected individuals compared to HIV-1 single-infected indivi-
duals. AIDS 2014; 28:949–957.
13. Ebsjornsson J, Mansson F, Kvist A, Isberg PE, Nowroozalizadeh S, Biague AJ, et al. Induction of HIV-
like disease progression by contemporaneous HIV-2 infection. N Engl J Med 2012; 367:224–232.
14. Hansson A, Sarri AD, Shea A, Jones N, Mboob S, Kanki P, et al. Distinct profile of T cell activation in HIV type 2 compared to HIV type 1 infection: differential mechanisms for immuno-
protection. AIDS Res Hum Retroviruses 2005; 21:791–798.
15. Deeks SG, Kitchen CM, Liu L, Gao H, Gasson R, Narvaez AB, et al. Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. Blood 2004; 104:942–947.
16. Lelieveldt A, Feldmann J, Jaye A, Cotten M, Dong T, McMichael A, et al. Direct relationship between virus load and systemic immune activation in HIV-2 infection. J Infect Dis 2010; 201:114–122.
17. Sousa AE, Carreno J, Meier-Schellersheim M, Grossman Z, Victorino RM. CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. J Immunol 2002; 169:3403–3406.
18. Giorgi JV, Detels R. T-cell subset alterations in HIV-infected homosexual men: NIAID Multicenter AIDS cohort study. Clin Immunol Immunopathol 1989; 52:10–18.
19. Douek DC, Roederer M, Koup RA. Emerging concepts in the immunopathogenesis of AIDS. Annu Rev Med 2009; 60:471–484.
20. Klatt NR, Chomont N, Douek DC, Deeks SG. Immune activation and HIV persistence: implications for curative approaches to HIV infection. Immuno Rev 2013; 254:326–342.
21. Lambin P, Desjoubet H, Debbia M, Fine JM, Muller YJ. Serum neopterin and beta-2-microglobulin in anti-HIV positive blood donors. Lancet 1986; 2:1216.
22. Popin J, Morgan G, Dunn D, Geva S, Mendy M, Gaye I, et al. HIV-2-induced immunosuppression among asymptomatic West African prostitutes: evidence that HIV-2 is pathogenic, but less so than HIV-1. AIDS 1991; 5:1163–1172.
23. Brenchley JM, Price DA, Schacker TW, Ascher TE, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med 2006; 12:1363–1371.
24. Nowroozalizadeh S, Mansson F, da Silva Z, Repits J, Dabo B, Pereira C, et al. Microbial translocation correlates with the severity of both HIV-1 and HIV-2 infections. J Infect Dis 2010; 201:1150–1154.
25. Lichtfuss GF, Cheng WJ, Farsakoglu Y, Paulovics G, Rajasuraj R, Velayudham P, et al. Virologically suppressed HIV patients show activation of NK cells and persistent innate immune activation. Clin Immunol 2012; 139:1–14.
26. Bisio F, Bozzano F, Marras F, Di Biagio A, Moretta L, De Maria R, et al. CD4+CD28−CD16+CD56+ NK cells: origin, function, and role in chronic viral disease. Trends Immunol 2010; 31:401–406.
27. Nuvor SV, van der Sande M, Rowland-Jones S, Whittle H, Jaye S, et al. Natural killer cell function is well preserved in asymptomatic HIV type 2 infected patients. J Exp Med 2004; 200:195–197.
28. Nuvor SV, van der Sande M, Rowland-Jones S, Whittle H, Jaye S, et al. Natural killer cell function is well preserved in asymptomatic HIV type 2 infected patients. J Exp Med 2004; 200:195–197.
29. Juno JA, Keynan Y, Fraske KR. Invariant NKT cells: regulation and function during viral infection. PLoS Pathog 2012; 8:e1002838.
30. Bjorkstrom NK, Ljunggren HG, Sandberg IK. CD56 negative NK cells: origin, function, and role in chronic viral disease. Trends Immunol 2010; 31:401–406.
31. Nuvor SV, van der Sande M, Rowland-Jones S, Whittle H, Jaye S, et al. Natural killer cell function is well preserved in asymptomatic human immunodeficiency virus type 2 (HIV-2) infection but similar to that of HIV-1 infection when CD4 T-cell counts fall. J Virol 2006; 80:2529–2538.
32. Sandberg IK, Bhardwaj N, Nixon DF. Dominant effector memory-lymphocyte characteristics, capacity for dynamic adaptive expansion, and sex bias in the innate Valpha24 NKT cell compartment. Eur J Immunol 2003; 33:588–596.
33. Breman PJ, Brigli M, Brenner MI. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nat Rev Immunol 2013; 13:101–117.
34. Bendelac A, Killeen N, Littman DR, Schwartz RH. A subset of CD4+ thymocytes selected by MHC class I molecules. Science 1994; 263:1774–1776.
35. Motsinger A, Haas DW, Stanic AK, Van Kaer L, Joyce S, Unutmaz D. CD1d-restricted human natural killer T cells are highly susceptible to human immunodeficiency virus 1 infection. J Exp Med 2002; 195:869–876.
36. Sandberg IK, Fast NM, Palacios EH, Fennelly G, Dobroszycki J, Palumbo P, et al. Selective loss of innate CD4(+)V alpha 24 natural killer T cells in human immunodeficiency virus infection. J Virol 2002; 76:7258–7263.
37. Moll M, Kuylenstierna C, Gonzalez VD, Andersson SK, Bosnjak Z, Sonnberg V, et al. Severe functional impairment and elevated PD-1 expression in CD1d-restricted NKT cells retained during chronic HIV-1 infection. Eur J Immunol 2009; 39:902–911.
38. van der Vliet HJ, van Blomberg BM, Haazen BD, Nishi N, Otto SA, van Benthen BH, et al. Selective decrease in circulating V alpha 24+V beta 11+NKT cells during HIV type 1 infection. J Immunol 2002; 168:1490–1495.
39. Mansson F, Biague A, da Silva ZI, Dias F, Nilsson LA, Andersson S, et al. Prevalence and incidence of HIV-1 and HIV-2 before, during and after a civil war in an occupational cohort in Guinea-Bissau, West Africa. AIDS 2009; 23:1575–1582.
40. Norrhen G, Andersson S, Naucier A, Dias F, Johansson I, Biberfeld G. HIV-1, HIV-2, HTLV-I/II and Treponema pallidum infections: incidence, prevalence, and HIV-2-associated mortality in an occupational cohort in Guinea-Bissau. J Acquir Immune Defic Syndr Hum Retrovirolog 1995; 9:422–428.
41. Norrhen G, da Silva Z, Biague A, Andersson S, Biberfeld G. Clinical progression in early and late stages of disease in a cohort of individuals infected with human immunodeficiency virus-2 in Guinea-Bissau. Scand J Infect Dis 2005; 35:265–272.
42. Ozakaya Sahin G, Holmgren B, Sheik-Khalil E, da Silva Z, Nielsen J, Nowroozalizadeh S, et al. Effect of complement on HIV-2 plasma antiviral activity is intratype specific and potent. J Virol 2013; 87:273–281.
43. Anglaret X, Diagbeuga S, Mertes E, Meda N, Verge-Valente V, Sylla-Koko F, et al. CD4+ T lymphocyte counts in HIV infection: are European standards applicable to African patients?. J Acquir Immune Defic Syndr Hum Retrovirol 1997; 14:361–367.
44. Norrhen G, da Silva Z, Biague A, Andersson S, Biberfeld G. Natural killer cell immunodeficiency in HIV disease is manifest by profoundly decreased numbers of CD16+CD56− cells and expansion of a population of CD16dimCD56− cells with low lytic activity. J Acquir Immune Defic Syndr Hum Retrovirol 1995; 10:331–336.
45. Ullum H, Gotzsche PC, Victoria J, Dickmeiss E, Velling P, Pedersen BK. Defective natural immunity: an early manifestation of human immunodeficiency virus infection. J Exp Med 1995; 182:799–799.
46. Hegenus A, Nyamweya S, Zhang Y, Jere J, Poss J, Mason A, et al. Protection versus pathology in aviremic and high viral load HIV-2 infection: the pivotal role of immune activation and T-cell kinetics. J Infect Dis 2014; 210:752–761.
47. Crawford A, Angelosanto JM, Kao C, Doering TA,icedi ZZ, Barnett BE, et al. Molecular and transcriptional basis of CD4+ T-cell dysfunction during chronic infection. Immunity 2014; 40:299–302.
48. Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. Curr Opin Immunol 2013; 25:161–167.
52. Daussy C, Faure F, Mayol K, Viel S, Gasteiger G, Charrier E, et al. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. J Exp Med 2014; 211:563–577.

53. Eidson M, Wahlstrom J, Beaulieu AM, Zaidi B, Carson SE, Crow PK, et al. Altered development of NKT cells, gammadelta T cells, CD8 T cells and NK cells in a PLZF deficient patient. PLoS One 2011; 6:e24441.

54. Gleimer M, von Boehmer H, Kreslavsky T. PLZF controls the expression of a limited number of genes essential for NKT cell function. Front Immunol 2012; 3:374.

55. Lazarevic V, Glimcher LH, Lord GM. T-bet: a bridge between innate and adaptive immunity. Nat Rev Immunol 2013; 13:777–789.

56. Hunt PW, Brenchley J, Sinclair E, McCune JM, Roland M, Page-Shafer K, et al. Relationship between T cell activation and CD4+ T cell count in HIV-seropositive individuals with undetectable plasma HIV RNA levels in the absence of therapy. J Infect Dis 2008; 197:126–133.

57. Naranbhai V, Altfeld M, Karim SS, Ndungu T, Karim QA, Carr WH. Changes in natural killer cell activation and function during primary HIV-1 infection. PLoS One 2013; 8:e53251.

58. Paiardini M, Muller-Trutwin M. HIV-associated chronic immune activation. Immunol Rev 2013; 254:78–101.

59. Nowroozalizadeh S, Mansson F, da Silva Z, Repits J, Dabo B, Pereira C, et al. Studies on toll-like receptor stimuli responsiveness in HIV-1 and HIV-2 infections. Cytokine 2009; 46:325–331.

60. Flexoridor R, Wilson B, Hou R, Landay A, Kessler H, Al-Harthi L. CD1d-restricted natural killer T cells are potent targets for human immunodeficiency virus infection. Immunology 2003; 108:3–9.

61. Alter G, Rihn S, Streeck H, Teigen N, Piechocka-Trocha A, Moss K, et al. Ligand-independent exhaustion of killer immunoglobulin-like receptor-positive CD8+ T cells in human immunodeficiency virus type 1 infection. J Virol 2008; 82:9668–9677.

62. Lunemann S, Malone DF, Grabowski J, Port K, Breziat V, Bremer B, et al. Effects of HDV infection and pegylated interferon alpha treatment on the natural killer cell compartment in chronically infected individuals. Gut 2015; 64:469–482.

63. Buggert M, Tauriainen J, Yamamoto T, Frederiksen J, Ivarsson MA, Michaelsson J, et al. T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection. PLoS Pathog 2014; 10:e1004251.

64. Hasley RB, Hong C, Li W, Friesen T, Nakamura Y, Kim GY, et al. HIV immune activation drives increased Eomes expression in memory CD8 T cells in association with transcriptional down-regulation of CD127. AIDS 2013; 27:1867–1877.

65. Alonzo ES, Sant’Angelo DB. Development of PLZF-expressing innate T cells. Curr Opin Immunol 2011; 23:220–227.

66. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, et al. The transcription factor PLZF directs the effector program of the NKT cell lineage. Immunity 2008; 29:391–403.

67. Gordon SM, Chaix J, Rupp LJ, Wu J, Madera S, Sun JC, et al. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. Immunity 2012; 36:55–67.

68. Matsuda JL, Zhang Q, Ndonye R, Richardson SK, Howell AR, Gapin L. T-bet concomitantly controls migration, survival, and effector functions during the development of Valpha14i NKT cells. Blood 2006; 107:2797–2805.

69. Townsend MJ, Weismann AS, Matsuda JL, Salomon R, Farnham PJ, Biron CA, et al. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. Immunity 2004; 20:477–494.