CONCISE COMMUNICATION

Impaired natural killer cell responses are associated with loss of the highly activated NKG2A$^+$CD57$^+$CD56^{dim} subset in HIV-1 subtype D infection in Uganda

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Objective: Of the predominant HIV-1 subtypes in Uganda, subtype D infection confers a worse prognosis. HIV-1 infection causes perturbations to natural killer (NK) cells, and yet these cells can exert immune pressure on the virus and influence clinical outcome. Here, we studied NK cell activation and function in Ugandans with chronic untreated HIV-1 subtype D infection in comparison to uninfected community matched controls.

Methods: Cryopreserved peripheral blood mononuclear cells (PBMCs) from 42 HIV-infected individuals and 28 HIV-negative controls were analysed using eight-colour flow cytometry. NK cell surface expression of CD16, CD56, CD57, HLA-DR and NKG2A were used to investigate activation, maturation and differentiation status. NK cell function was evaluated by measuring interferon-gamma (IFNγ) production in response to K562 cells, or interleukin (IL)-12 and IL-18.

Results: CD56^{dim} NK cells from HIV-infected individuals produced less IFNγ in response to IL-12 and IL-18 than did CD56^{dim} NK cells from uninfected controls. Infected individuals had lower levels of CD56^{dim} NK cells coexpressing the differentiation markers NKG2A and CD57 than controls. In addition, their NKG2A$^+$CD57$^+$ CD56^{dim} NK cells displayed elevated activation levels as assessed by HLA-DR expression. Cytokine-induced IFNγ production correlated directly with coexpression of CD57 and NKG2A on CD56^{dim} NK cells.

Conclusion: HIV-1 subtype D infection is associated with impaired NK cell responsiveness to cytokines, decline of the NKG2A$^+$CD57$^+$ CD56^{dim} NK cell subset, as well as

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elevated activation in this subset. These alterations within the NK cell compartment may contribute to immunopathogenesis of HIV-1 subtype D infection in Ugandans.

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Keywords: AIDS, CD57, HIV-1, immune activation, natural killer cells, NKG2A, subtype D

Introduction

The Ugandan HIV-1 epidemic, with a prevalence of 7.3% [1], is predominantly composed of subtype A, D and recombinant viruses [2,3]. HIV-1 subtype affects the rate of disease progression [4], yet it is not clear why. Subtype D is associated with faster progression to AIDS ([5], reviewed in [6,7]), more profound loss of $CD4^+$ T cells [5,8] and decreased frequency of invariant natural killer T cells [9], as compared with subtype A. Further studies of subtype D infection are warranted to understand subtype-dependent differences in HIV-1 immunopathogenesis.

Natural killer (NK) cells are an integral part of the innate immune response to viruses [10]. HIV-1 infection, however, contributes to a range of changes in NK cell phenotype and function as early as primary infection [11–15]. In addition, HIV-1 can acquire escape mutations under NK cell immune pressure [16], although certain killer immunoglobulin-like receptor (KIR) alleles, primarily expressed by NK cells, are associated with slower progression to AIDS [17]. Furthermore, the KIR repertoire of the NK cell compartment adapts and changes during the response to HIV-1 infection [18,19].

NK cells interact with dendritic cells and T cells to shape the immune response to infection. This cross-talk involves the activation of NK cells via interleukin (IL)-12 and IL-18 to release type II interferon, which in turn matures dendritic cells to adequately prime T-cell responses [20,21]. Disruption of the NK–dendritic cell communication may adversely impact immune control of HIV [22,23]. Cytokine-activated NK cells can migrate to lymph nodes [24], and directly activate T cells [25]. Cell-surface receptors such as NKG2A and CD57 can be used to describe $CD56^{dim}$ NK cell differentiation [26]. In the present study, we investigated NK cell activation, differentiation and function in Ugandans with chronic untreated HIV-1 subtype D-infection.

Materials and methods

Study cohort and samples

Study participants were randomly selected from a prospective community-based cohort to characterize HIV-1 infection in Rakai, Uganda, from 1998 until 2004 [5] prior to the availability of antiretroviral therapy in this setting. Cryopreserved peripheral blood mononuclear cells (PBMCs) from 42 treatment-naive individuals infected with HIV-1 subtype D and 28 community-matched HIV-uninfected controls were selected for study (Table 1). Walter Reed Army Institute of Research, Human Subjects Protection Branch (WRAIR#1428), as well as the Uganda National Council of Science and Technology (HS413) approved this study, and all participants provided written consent for participation and for use of stored samples. Plasma viral load was measured using Amplicor HIV-1 Monitor test version 1.5 (Roche Diagnostics, Indianapolis, Indiana, USA). HIV-1 subtypes were determined using the previously described multiregion hybridization assay ($MHA_{acd,}$ [27]). The FACS MultiTEST IMK Kit (BD Biosciences, San Jose, California, USA) was used to enumerate lymphocyte subsets on a dual-laser FACSCalibur (BD Biosciences).

Flow cytometry

Whole blood collected in acid citrate dextrose anticoagulant was processed within 8 h and PBMC cryopreserved at −130°C in liquid nitrogen, as previously described [28]. PBMCs were thawed and washed in RPMI medium containing 10% foetal bovine serum, 5% L-glutamine, 1% penicillin/streptomycin and 5% HEPES, and cell concentration and viability determined using

Table 1. Descriptive statistics for study population.

Characteristic	HIV-negative ($n = 28$)	HIV-1 subtype D ($n = 42$)
Age, median (years, range)	36 (22–48)	31 (19–54)
Sex, no (percentage)		
Female	14 (50%)	28 (67%)
Male	14 (50%)	14 (33%)
$CD4^+$ T-cell absolute count, median (cells/μl, range)	1013 (527–1659)	475 (85–1336)
Viral load, median (copies/ml, range)[a]	NA	54 157 (562–1 484 450)

NA, not applicable. [a]Viral load measured by Roche Amplicor Monitor v1.5, limit of detection 400 copies/ml.

Guava Viacount reagent and the Guava Personal Cell Analysis System (EMD Millipore, Billerica, Massachusetts, USA) [28]. Samples were stained in 96-well U-bottom plates at 4°C for 30 min in the dark [12]. Aqua Live-Dead stain (Life Technologies Corporation, Carlsbad, California, USA) was used to discriminate viable cells. Commercially available mAbs included anti-CD3, anti-CD14 and anti-CD19 (all on APC-H7), anti-CD56 PE-Cy7, anti-Ki67 PE and anti-HLA-DR V450 (BD Biosciences). Additional mAbs included anti-NKG2A APC (Beckman Coulter, Inc., Brea, California, USA), anti-CD57 FITC (BioLegend, San Diego, California, USA) and anti-perforin PE (eBioscience, San Diego, California, USA). Ki67 and perforin were stained intracellularly after permeabilization using BD Perm/Wash (BD Biosciences). To assess NK cell function, thawed PBMCs were cultured overnight, in the presence of brefeldin A (BD Biosciences), with 10 ng/ml rIL-12 (R&D systems) as well as 10 ng/ml rIL-18 (MBL), or the MHCnull K562 cell line (at an E:T of 5:1), or media alone [12]. Samples were fixed, washed, permeabilized and stained with anti-IFNγ PE (BD Biosciences). All samples were acquired on an eight-colour BD FACSCanto II (BD Biosciences) and analysed using FlowJo version 9.5.3 (Tree Star, Ashland, Oregon, USA).

Statistical analysis

Graph Pad Prism version 6.0a for Mac OSX was used for statistical analysis (GraphPad Software, La Jolla, California, USA). Differences between groups were analysed using the Mann–Whitney test and associations were determined using Spearman's rank correlation. *P* values less than 0.05 were considered statistically significant.

Results

Impaired CD56^{dim} natural killer cell response to interleukin-12/18 stimulation in HIV-1 subtype D infection

NK cell phenotype and function were studied from the PBMCs of 42 untreated HIV-1 subtype D-infected individuals and 28 community-matched uninfected controls (Table 1). Individuals had a median viral load of 54 147 copies/ml and median CD4$^+$ absolute cell count of 475 cells/μl. NK cells were distinguished on the basis of live, CD3$^-$CD14$^-$CD19$^-$ and CD56$^+$ gating (Fig. 1a). After stimulation with IL-12 and IL-18, CD56^{dim} NK cells in HIV-infected individuals produced less IFNγ than those from uninfected controls ($P = 0.026$, Fig. 1b). The overall trend towards lower absolute counts of CD56^{dim} NK cells ($P = 0.07$, data not shown) in HIV infection enhanced this pattern in absolute count terms with diminished counts of IFNγ+ CD56^{dim} NK cells in infected individuals ($P < 0.001$, Fig. 1c). In contrast, IFNγ production in CD56^{dim} NK cells in HIV-infected individuals was unimpaired after K562 stimulation ($P = 0.5$, Fig. 1d), although the overall trend towards lower absolute counts of CD56^{dim} NK cells led to a lower representation of cells responding to K562 measured as an absolute count ($P = 0.036$, Fig. 1e). Expression of Ki67 and the cytolytic protein perforin were similar regardless of infection status (data not shown).

The NKG2A$^+$CD57$^+$CD56^{dim} natural killer cell subset is activated and declines in subtype D infection

To characterize defects in NK cells in more detail, we studied the phenotype of the CD56^{dim} NK cells using the activation marker HLA-DR, inhibitory receptor NKG2A and the terminal differentiation marker CD57. Individuals had a lower representation of NKG2A$^+$CD57$^+$ CD56^{dim} NK cells than the healthy controls measured both as a percentage of CD56^{dim} NK cells ($P = 0.002$, Fig. 1f) and as an absolute count of this subset of CD56^{dim} NK cells ($P < 0.001$, Fig. 1g). The proportions of cells defined by other combinations of these markers (NKG2A$^+$CD57$^-$, NKG2A$^-$CD57$^+$ and NKG2A$^-$CD57$^-$) did not change significantly between HIV-1-infected and uninfected individuals, suggesting that the relative decrease in NKG2A$^+$CD57$^+$ cells was not directly related to an increase in one of the other subsets (data not shown). Furthermore, the NKG2A$^+$CD57$^+$CD56^{dim} NK cell subset was more activated, as determined by HLA-DR expression, in a subset of 18 of the individuals when compared with 19 controls ($P = 0.011$; Fig. 1h).

NKG2A$^+$CD57$^+$CD56^{dim} natural killer cell frequency correlates with CD56^{dim} interferon-gamma production

We next investigated whether the decline in NKG2A$^+$ CD57$^+$ NK cells was associated with the functional impairment of CD56^{dim} NK cells in HIV-1 subtype D-infected individuals. The frequency of NKG2A$^+$ CD57$^+$CD56^{dim} NK cells correlated directly with CD56^{dim} IFNγ production in response to IL-12 as well as IL-18 stimulation (rho = 0.568, $P < 0.001$; Fig. 1i), but not K562 stimulation (rho = 0.030, $P = 0.85$; Fig. 1j). This association was not observed in controls (rho = −0.080, $P = 0.68$; data not shown). Surprisingly, cytokine-induced IFNγ production in NKG2A$^+$CD57$^+$ CD56^{dim} NK cells did not differ between cases and controls (Fig. 1k). This suggests that the functional capacity of NKG2A$^+$CD57$^+$ CD56^{dim} NK cells may not be directly affected on a per-cell basis, and that their decline may be associated with the functional capacity of the global CD56^{dim} NK cell compartment. Of note, these phenotypic and functional parameters did not correlate with CD4$^+$ cell count or viral load (data not shown).

Discussion

To better understand the innate immunology of HIV-1 subtype D infection in Rakai, Uganda, we studied NK cell responses to IL-12 as well as IL-18 stimulation.

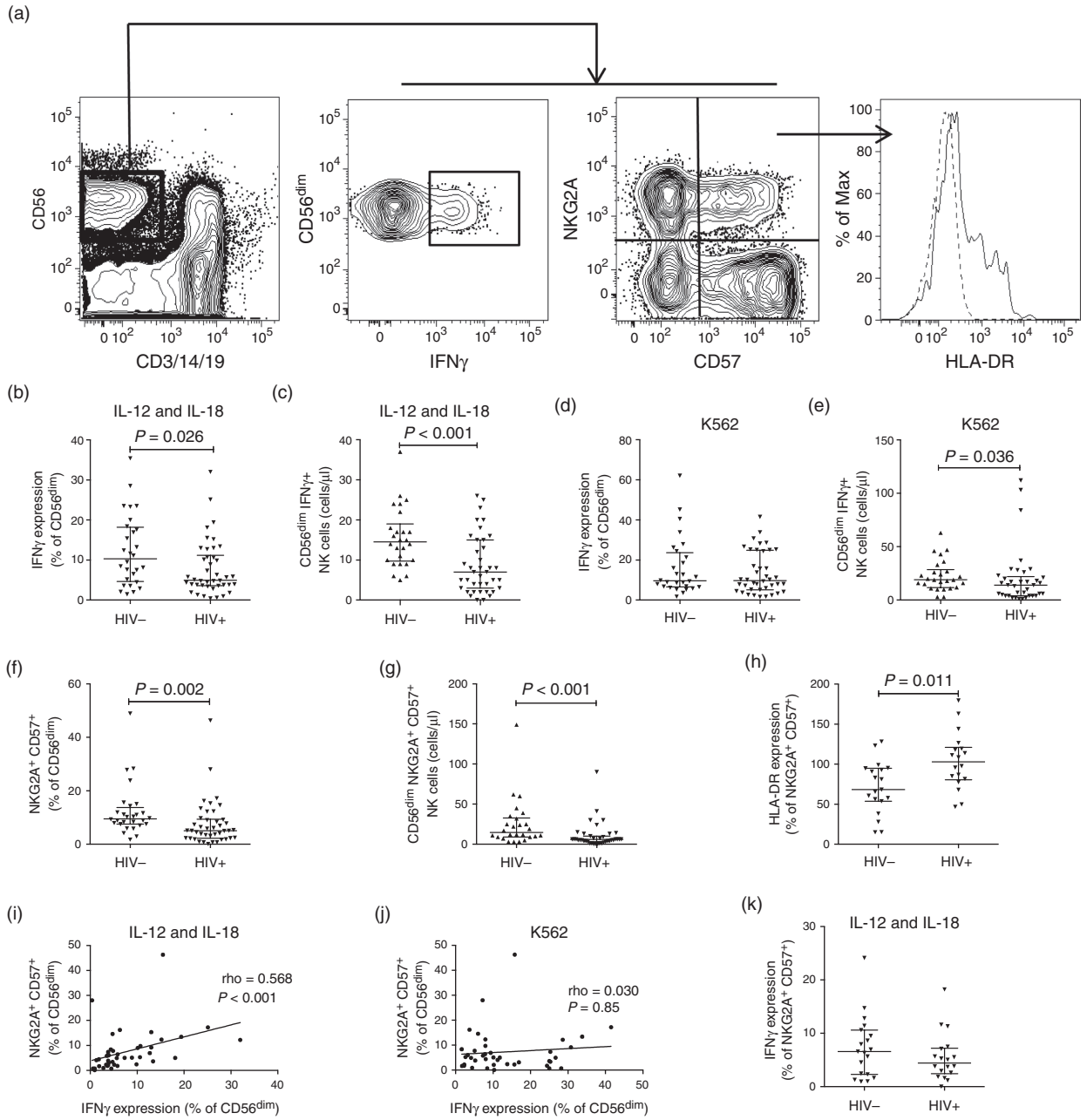

Fig. 1. Characteristics of natural killer cells in HIV-1 subtype D infection. Contour plots, with outliers, illustrating the gating strategy used to identify $CD56^{dim}$ NK cells expressing IFNγ, NKG2A, CD57 and HLA-DR (a). The dashed histogram is the FMO and the continuous line shows HLA-DR expression. Scatter dot plots comparing the median frequencies and interquartile ranges of $CD56^{dim}$ NK cells expressing IFNγ in HIV-infection when stimulated with IL-12 and IL-18, expressed as a percentage (b) or an absolute count (c). Percentage (d) and absolute count (e) responses after stimulation with K562. Levels of $NKG2A^{+}CD57^{+}$ cells as a percentage (f) and absolute count (g). Activation levels in $NKG2A^{+}CD57^{+}$ $CD56^{dim}$ NK cells (h). Frequency of the $NKG2A^{+}CD57^{+}$ $CD56^{dim}$ subset correlated with the frequency of $CD56^{dim}$ NK cells expressing IFNγ when stimulated with IL-12 and IL-18 (i), or K562 (j). IFNγ expression in $NKG2A^{+}CD57^{+}CD56^{dim}$ NK cells after IL-12 and IL-18 stimulation (k).

$CD56^{dim}$ NK cells from HIV-positive individuals produced less IFNγ in response to cytokine stimulation, as compared with uninfected controls. This is in line with previous findings from a US cohort, despite the geographic, host and viral disparity between these populations [29]. Expression of the inhibitory receptor NKG2A and the terminal differentiation marker CD57 together identified a subset of $CD56^{dim}$ NK cells that was reduced in HIV-1 subtype D infection. These $NKG2A^{+}CD57^{+}$ cells displayed elevated activation levels in HIV-positive individuals, measured by HLA-DR, and their frequency correlated directly with the proportion of

IFNγ expressing $CD56^{dim}$ NK cells in response to cytokine stimulation.

$CD56^{dim}$ NK cells differentiate from high expression of NKG2A in the absence of KIRs and CD57 expression, to a terminally differentiated $NKG2A^{-}KIR^{+}CD57^{+}$ $CD56^{dim}$ subset, with those expressing CD57 losing their ability to respond to cytokine stimulation [26]. Previous studies have indicated that NKG2A expression correlates with the IFNγ response to IL-12 as well as IL-18 cytokine stimulation [30], and that CD57 expression reflects the cytolytic potential of NK cells in the form of perforin and granzyme expression [31]. $NKG2A^{+}CD57^{+}CD56^{dim}$ cells may be in an intermediate differentiation phase, retaining responses to cytokine stimulation and in transition to a more differentiated phenotype [24]. Our finding that the decline of the $NKG2A^{+}CD57^{+}CD56^{dim}$ NK cell subset is associated with impaired NK cell responses to cytokine stimulation suggests a possible important role for this subset in NK cell homeostasis and function.

Pathogen-associated maturational skewing of NK cells has been observed previously. NK cells reconstituting in patients after umbilical cord blood transplantation mature more rapidly in patients infected with cytomegalovirus [32], a pattern also seen in primary HIV infection [33]. Hong *et al.* [34] observed a decline of less differentiated NK cells in chronic untreated HIV-1 infection. In addition, Marras *et al.* [35] recently showed that activation, as measured by HLA-DR, of $CD56^{dim}$ NK cells in HIV-1 viremic controllers is associated with differentiation to an $NKG2A^{-}CD57^{+}$ effector phenotype. Chronic viral infection has also been associated with a defect in miR-155, which is important for IFNγ production [36], maturation and expansion of IL-12 and IL-18 activated NK cells [37]. In this context, the decline in the activated $NKG2A^{+}CD57^{+}CD56^{dim}$ subset could be a consequence of HIV-driven rapid maturation [34] that is not accompanied by expansion of the effector NK cell subset [32,37]. This may reduce the number of NK cells available to produce IFNγ.

Wright *et al.* [38] found lower plasma IFNγ concentration in 'late stage' subtype D-infected patients than in subtype A-infected Ugandans at a similar disease stage. Others have also observed that IFNγ secretion by NK cells declines during HIV-1 infection [39] and is not restored by antiretroviral therapy, despite recovery of phenotypically mature NK cells [29]. These observations, together with the lack of association of the IFNγ 'defect' with $CD4^{+}$ cell count in the present study, may suggest that even relatively healthy HIV-infected individuals have damaged NK cell compartments. In summary, our findings support the notion that HIV-1 infection drives changes in NK cell maturation, with an altered distribution of maturational subsets, elevated expression of activation markers and lower responsiveness to cytokine stimulus.

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Conflicts of interest

The authors declare no financial conflict of interest.

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