High frequencies of polyfunctional CD8\(^+\) NK cells in chronic HIV-1 infection are associated with slower disease progression

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Abstract

Summary: Natural killer (NK) cells are effector and regulatory innate immune cells and play a critical role in the first line of defense against various viral infections. Although previous reports indicated vital contributions of NK cells to HIV-1 immune control, non-genetic NK cell parameters directly associated with slower disease progression have not been defined yet.

In a longitudinal, retrospective study of 117 untreated HIV-infected subjects we show that higher frequencies as well as absolute numbers of CD8^+CD3^- lymphocytes are linked to delayed HIV-1 disease progression. We show that the majority of these cells are well-described blood NK cells. In a subsequent cross-sectional study we demonstrate a significant loss of CD8^- NK cells in untreated HIV-infected individuals, which correlated with HIV viral loads and inversely correlated with CD4^+ T cell counts. CD8^- NK cells had modestly higher frequencies of CD57-expressing cells compared to CD8^- cells but no differences in the expression of a number of activating and inhibiting NK cell receptors. However, CD8^+ NK cells exhibited a more functional profile as detected by cytokine production and degranulation.

Importance: We demonstrate that the frequency of highly functional CD8^+ NK cells is inversely associated with HIV-related disease markers and linked with delayed disease progression. These results thus indicate that CD8^- NK cells represent a novel NK cell-derived, innate immune correlate with an improved clinical outcome in HIV infection.
Introduction

NK cells are traditionally seen as innate immune cells constituting a first line of immune defense against malignant cells and viruses (1, 2). The recognition of virally infected cells is mediated by an arsenal of inhibitory and activating receptors and the sum of the signals mediated by these determines the functional activity of NK cells (2-4). Different subsets of NK cells have been described in the peripheral blood of humans (3, 5). The majority of peripheral blood NK cells are CD56\textsuperscript{dim}CD16\textsuperscript{+} cells whereas lymph node resident NK cells are predominantly CD56\textsuperscript{bright} NK cells (3, 5-7). Roughly 30% of peripheral blood NK cells express the CD8\textalpha homo-dimer and these cells were shown to exhibit better survival during target cell killing (8, 9).

Genetic studies at the population level provided evidence for a protective role of NK cells in HIV-1 infection. For instance, certain KIR3DL1 alleles in the context of HLA-Bw4 were shown to exert a strong protective influence during HIV infection (10). HIV-infected individuals expressing KIR3DS1 in conjunction with HLA-Bw4-80I exhibited a considerably slower progression to AIDS (11). Notably, these epidemiological studies were supported by subsequent functional NK cell studies and by the observation of NK cell-mediated sieve effects in carriers of KIR3DS1 and HLA-Bw4-80I (12-14). Furthermore, KIR2DL2-expressing NK cells were suggested to mediate significant immune pressure against HIV as evidenced by selected amino acid polymorphisms in the viral sequence leading to a decreased ability of NK cells to kill virus-infected CD4\textsuperscript{+} T cells (15). These data thus indicate that NK cells are potentially important contributors to the host immune defense against HIV-1.

However, to this day non-genetic NK cell parameters associated with a slower clinical HIV-1 disease progression are unknown.

NK cells are also subject to profound alterations in chronic HIV-1 infection. A number of reports have demonstrated phenotypic and functional changes in peripheral blood NK cells.
during HIV-1 infection in humans (16-21). Progressive HIV-1 infection is associated with a decline in cytotoxic CD56dimCD16+ NK cells and an expansion of dysfunctional CD56 CD16+ NK cells (22, 23). We have previously shown a decline of less differentiated and functionally more potent CD56dimCD16+ NK cells, which are either CD57- or CD57dim (20). We furthermore demonstrated significant correlations of HIV viral loads and decrease of CD4+ T cell counts with a loss of CCR7 on CD56bright NK cells indicating that a NK cell-derived parameter can robustly correlate with HIV-related disease markers (18). Notably, NK cells from patients able to spontaneously control HIV replication and long-term non-progressors maintained unchanged properties, especially with regards to their ability to express natural cytotoxicity receptors (24).

Here, we performed a retrospective study in 117 untreated HIV-infected patients and found that relative numbers and absolute counts of CD8+CD3- lymphocytes and NK cells were significantly correlated with slower clinical progression to AIDS. Subsequent analysis revealed that the majority of CD8+CD3- cells were CD56-expressing NK cells. Loss of CD8+ NK cells was significantly correlated with HIV-1 plasma viral loads and inversely correlated with CD4+ T cell counts as well as the CD4/CD8 T cell ratio. Furthermore, CD8+ NK cells exhibited a more functional profile compared to their CD8- counterpart. These results therefore indicate that the frequency of highly functional CD8+ NK cells represents a robust correlate for delayed disease progression to AIDS in chronic HIV infection.
Materials and Methods

**Study subjects.** The data presented in this study is derived from a cohort of altogether 162 HIV-seropositive patients and 15 uninfected control subjects. From the 162 HIV-infected patients, 117 untreated subjects with CD4 T cell counts above 500 cells/μl were longitudinally followed at our institution for up to 90 months with a median follow-up time of 30.6 months (interquartile range 18.5 to 50.4). A description of this longitudinal patient cohort is provided in Table 1. Peripheral blood samples from 60 untreated HIV-seropositive subjects chosen based on sample availability were analyzed in a cross-sectional study. 25 of these 60 patients were part of the longitudinal study but none of these samples were taken at baseline or endpoint. Blood samples for the cross-sectional study were taken at random time points during patient’s visits after study recruitment and thus encompass various clinical stages. To study the effect of ART on CD8 expression on NK cells, samples from 28 patients at least one year on ART with suppressed viral loads were analyzed (8/28 overlap with longitudinal cohort). We obtained samples from 21 individuals before and after the initiation of ART (9/21 overlap with longitudinal cohort). All study subjects were recruited at the HIV outpatient clinic of the Medizinische Hochschule Hannover (MHH) and gave written, informed consent prior to their participation. The study was approved by the local ethics committee (Votum der Ethikkommission der MHH No. 3150). Plasma HIV-1 RNA levels was measured using the COBAS TaqMan HIV-1 test (Roche Diagnostics) with a lower limit of detection of 34 copies/mL. NK cell, CD4+ and CD8+ T cell as well as CD8+CD3- lymphocyte counts were routinely determined by a flow cytometry-based assay using CYTO-STAT tetra CHROME (Beckman Coulter).

**Blood sample processing.** Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood using Ficoll (Biochrom) density gradient centrifugation. Cells were washed three
times with PBS and aliquots of $10^7$ PBMCs were cryopreserved in heat-inactivated FCS supplemented with 10% dimethyl sulfoxide (Merck).

Phenotypic analysis of NK cells by flow cytometry. PBMCs were thawed and washed in PBS supplemented with FCS. Staining and flow cytometric analyses were performed as described before (19, 25). Intracellular expression of perforin, granzyme B and Ki-67 was analyzed ex vivo in unstimulated NK cells using ‘Fix and Perm’ kit (Life Technologies) following the manufacturer’s protocol. Following monoclonal antibodies were employed in our study: anti-CD19 PerCP, anti-CD14 PerCP, anti-CD3 PerCP, anti-CD16 APC-H7, anti-CD94 APC, anti-NKG2D APC, purified anti-NKp46, anti-NKp80-Biotin, purified anti-CD85j, anti-CD2 Biotin, anti-CD57 FITC, anti-HLA-DR V500, anti-perforin PE, anti-granzyme B AlexaFluor700, anti-Ki-67 Alexa Fluor 647 (BD Biosciences), anti-CD8 FITC, anti-KIR2DL2/DL3 APC (BioLegend), anti-CD3 ECD, anti-CD62L ECD, anti-NKp30 PE, and anti-NKG2A PE (Beckman Coulter). Secondary staining was performed using Streptavidin Alexa Fluor 700 and Goat Anti-Mouse Pacific blue (Life Technologies). Stained cells were immediately run on a LSR II SORP flow cytometer (BD Biosciences). At least 1,000 gated events had to be acquired for CD8$^+$ and CD8$^-$ NK cells for samples to be evaluable for phenotypic analyses.

Degranulation and intracellular cytokine staining assay. Functional NK cell assays were performed as described previously (19, 26). Briefly, $10^5$ sorted NK cells were stimulated with 100 ng/ml IL-12, 10 ng/ml IL-15 and/or K562 cells at an E:T ratio of 2:1. After 1 hour of culture, 1µg/mL Brefeldin A (Sigma), GolgiPlug and anti-CD107a PE (BD Biosciences) were added and cells were cultured for another 5 hours. Anti-IFN-γ Pacific-Blue (Biolegend), anti-TNF-α Alexa Fluor 700 and anti-MIP-1β PE (BD Biosciences) were used to detect intracellular expression of cytokines after fixation and permeabilization.
Univariate and multivariate survival analyses. Survival was calculated from the date of recruitment to the date of end point, that is, disease progression. To define survival comparison groups, patients were ranked according their absolute and relative CD8⁺CD3⁻ cell counts or absolute and relative NK cell counts and subjects belonging to the first tertile were compared to patients belonging to the second and third tertiles. Initially, study end point was either defined as initiation of ART or a drop of CD4⁺ T cell counts below 200 cells/µl. Since ART was initiated at various CD4⁺ T cell counts (316 cells/µl median, 251 to 433.5 interquartile range) we sought to answer the question whether there was a potential ART bias. Thus, further survival analyses were performed using ART only as end point. Individuals who did not achieve their end point were censored on their last clinic visit date or latest after 90 months. GraphPad Prism (version 5.0) was used to perform log-rank tests. Cox proportional hazards regression (backward conditional) was calculated using IBM SPSS Statistics 20.

Included in the analysis were continuous variables: percentages of CD4⁺ T cells, CD8⁺ T cells, CD8⁺CD3⁻ cells, NK cells, CD4⁺/CD8⁺ T cell ratio and age. Categorical variables were gender (females vs. male), ethnicity (Caucasian, African, Asian), viral copy numbers (per 100,000 copy/ml increment) and absolute counts of CD4⁺ T cells, CD8⁺ T cells, CD8⁺CD3⁻ cells and NK cells (per 100/µl increment).

Further statistical analyses. For all other statistical analyses as well as graph plotting GraphPad Prism was employed. Spearman rank correlation analysis was used to assess correlations between the frequency of CD8⁺ NK cells and HIV disease parameters. Pestle and Spice software were provided by the NIH (27) and used to visualize co-expression patterns of surface markers and multifunctional NK cell responses. Unless otherwise indicated, unpaired, two-tailed t test when comparing two groups or One-way ANOVA followed by Tukey post
test when comparing more than two groups were performed and $P$ values of less than 0.05 were considered significant.
Results

High frequencies of CD8\(^+\)CD3\(^-\) lymphocytes are associated with slower HIV-1 disease progression

A cohort of 117 untreated, chronically HIV-infected subjects was followed for up to 90 months at our outpatient clinic. At time of inclusion, the patients had a baseline CD4\(^+\) T cell count of over 500 n/\(\mu\)l. The frequency as well as absolute numbers of CD8\(^-\)CD3\(^-\) cells were routinely determined from all HIV-infected patients at our outpatient clinic. To assess the association of the frequencies of CD8\(^-\)CD3\(^-\) lymphocytes with HIV disease progression we ranked the cohort according to their absolute or relative CD8\(^-\)CD3\(^-\) cell counts. We compared patients belonging to the first tertile with high (> 83 cells/\(\mu\)l or > 3.9% of lymphocytes, \(n = 39\)) CD8\(^-\)CD3\(^-\) cell counts to patients belonging to the second and third tertiles with low (<83 cells/\(\mu\)l or < 3.9% of lymphocytes, \(n = 78\)) CD8\(^-\)CD3\(^-\) cell counts at baseline. We initially defined disease progression as a loss of CD4\(^+\) T cells below 200 cells/\(\mu\)l or initiation of ART. We identified a significantly slower disease progression in the cohort with high numbers of CD8\(^-\)CD3\(^-\) cells compared to patients with lower numbers of CD8\(^-\)CD3\(^-\) cells (Fig. 1A, B and Suppl. Table 1A).

ART was initiated at various CD4\(^+\) T cell counts ranging from 202 to 619 cells/\(\mu\)l (median 251 cells/\(\mu\)l). To examine whether there was a potential bias caused by ART we repeated the survival analysis after censoring patients with CD4\(^+\) T cell counts below 200 cells/\(\mu\)l at end point, thus using ART only as end point. Again, we found significantly delayed HIV disease progression in patients with high absolute and relative CD8\(^-\)CD3\(^-\) lymphocytes (Fig. 1C, D and Suppl. Table 1A). We furthermore ranked all patients who had reached their end point by ART initiation according to their CD4\(^+\) T cell counts at end point. After censoring subjects belonging to the first tertile (ART initiation at CD4\(^+\) T cells counts > 359, \(n = 12\)), patients with high CD8\(^-\)CD3\(^-\) lymphocyte counts still exhibited prolonged survival (Fig. 1E). Since there were an unusually high number of elite controllers in our cohort (defined as...
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undetectable viral loads during at least three visits during a period of 12 months or longer) we sought to address if these could potentially influence our survival analysis. After exclusion of these 7 patients we still found the survival difference to be statistically significant (Fig. 1F).

We furthermore used Cox proportional hazards regression (backward conditional, upper tertile vs. lower two tertiles) adjusting for age, gender, ethnicity, CD4+ T cells, CD8+ T cells, CD4/CD8 ratio and CD8'+CD3' cell count and percent and viral load. The final Cox model for the combined endpoint of either ART initiation or a CD4+ T cell drop below 200 counts/µl, the ART only end point and a further analysis excluding elite controllers included percentages of CD4+ T cells, CD8'+CD3' cell count and viral load as independent prognostic factors (Suppl. Table 1B). For the combined endpoint higher CD4+ percentage (odds ratio = 0.93), higher CD8'+CD3' count (odds ratio = 0.46 / increase by 100) and lower viral load (odds ratio = 1.42 / increase by 100,000) were associated with slower progression to endpoint. We thus show that high numbers of CD8'+CD3' cells were independently associated with an improved clinical outcome while controlling for the above-mentioned factors (Suppl. Table 1B).

CD8'+CD3' cells are mostly CD56-expressing NK cells

Having shown that higher frequencies of CD8'+CD3' lymphocytes are associated with a prolonged AIDS-free survival, we next sought to characterize these cells. Since CD8 in conjunction with other cell surface antigens is frequently used to define nonhuman primate NK cells (28), we hypothesized that the majority of CD8'+CD3' cells are NK cells. To address this question, we analyzed randomly sampled PBMC specimen derived from 37 HIV patients. We also included 15 HIV-seronegative control subjects. After gating on CD8'+CD3' lymphocytes, a subsequent analysis of CD56 and CD16 expression revealed that the majority of these cells are previously well-defined peripheral blood NK cell subsets, namely, CD56^{bright} (4.7% ± 2.2% SD and 6.3% ± 5.4% SD, HIV patients and control subjects, respectively), CD56^{dim}CD16' (74% ± 11.6% SD and 50% ± 22.9% SD) and CD56 CD16' (6.7% ± 4.1% SD
and 19.3% ± 12.2% SD) cells (Fig. 2A, B). We and others previously reported a decrease of CD56<sup>dim</sup>CD16<sup>+</sup> NK cells and an expansion of less functional CD56 CD16<sup>+</sup> NK cells in chronic HIV-1 infection (17, 19, 20). These NK cell subset alterations were also evident in the distribution of NK cell subsets within CD8<sup>+</sup>CD3<sup>−</sup> cells (Fig. 2B).

Notably, among HIV-seropositive subjects the portion of CD56 CD16<sup>−</sup> cells was larger compared to uninfected blood donors. Upon closer examination we found that these cells were mostly negative for NK cell markers, such as NKp46 and NKp80, and the majority of these cells had high expression levels of HLA-DR strongly suggesting these were not NK cells (data not shown). Importantly, a high percentage of these cells did not translate into high absolute numbers of CD8<sup>+</sup>CD3<sup>−</sup>CD56<sup>−</sup>C16<sup>−</sup> cells. For example, one patient with 70% CD56<sup>−</sup>CD16<sup>−</sup> of CD8<sup>+</sup>CD3<sup>−</sup> lymphocytes had very low numbers of CD8<sup>+</sup>CD3<sup>−</sup> cells (0.3% of lymphocytes). Thus, a high percentage of CD56 CD16<sup>−</sup> cells among the CD8<sup>+</sup>CD3<sup>−</sup> lymphocytes was in general reflective of lower numbers of the parent population.

Since the majority of CD8<sup>+</sup>CD3<sup>−</sup> cells were thus ‘conventional’ blood NK cells, we sought to answer the question whether there were comparable associations of high frequencies of NK cells with slower HIV-1 disease progression. Absolute and relative numbers of NK cells defined as CD3<sup>−</sup>, CD16<sup>+</sup> and/or CD56<sup>+</sup> cells were determined for all HIV-seropositive patients as part of the routine clinical analysis. We thus ranked the cohort according to their absolute and relative NK cell numbers and compared the survival of patients belonging to the first tertile with patients belonging to the second and third tertiles. Patients with high absolute NK cell counts (>230 cells/μl) were associated with a delayed onset of disease progression compared to subjects with lower NK cell numbers (Fig. 2C, Suppl. Table 1A). Elevated relative NK cell frequencies (>10,3%) were associated with a modestly improved clinical outcome (Fig. 2D).

We next sought to determine whether CD8<sup>+</sup>CD3<sup>−</sup> cells or overall NK cells primarily correlated with delayed HIV-1 disease progression. To this end, we either subtracted absolute
counts of CD8⁺CD3⁻ cells from the absolute counts of total NK cells or subtracted the percentage of CD8⁺CD3⁻ cells from the percentage of total NK cells. With the caveat that there was some variability in the frequency of CD56⁻CD16⁻ non-NK cells within CD8⁺CD3⁻ lymphocytes, this approach allowed us to determine an approximation of absolute or relative numbers of CD8⁻ NK cells. We subsequently performed a survival analysis comparing HIV-1 seropositive patients with either high (>80 cells/ul or >4%) or low (<80 cells/ul or <4%) NK cells devoid of CD8. Interestingly, we found that neither frequencies nor absolute counts of CD8⁺CD3⁻ cell-subtracted NK cells were associated with slower HIV-1 disease progression (Fig. 2E-F) suggesting that CD8⁺ but not CD8⁻ NK cells represent a correlate for an improved HIV-1 disease outcome.

**CD8⁺ NK cells correlate with clinical parameters in untreated, chronic HIV-1 infection**

Given that CD8⁺CD3⁻ cells predominantly consist of CD8⁺ NK cells expressing CD56 and/or CD16 and having shown that a high frequency of these cells correlated with delayed HIV-1 disease progression, we next analyzed the frequency of CD8-expressing cells in total peripheral blood NK cells. The clinical flow cytometric data, which was the basis for our survival analyses, allowed a solid enumeration of lymphocyte subsets. However, as a consequence of the limited number of parameters employed in these analyses (4-5 colors), residual contaminations by non-NK cells cannot be ruled out. Whereas these potential contaminations should not affect overall numbering of NK cells much, there could be an impact on downstream phenotypic and functional studies. Thus, for all subsequent analyses, we performed 9- to 10-color polychromatic flow cytometry and were able to employ an improved gating strategy. We excluded doublets and dead cells and defined NK cells as CD3⁻, CD14⁻, CD19⁻, CD56⁺ and/or CD16⁺ lymphocytes as depicted in Suppl. Fig. 1A. In addition, possible myeloid cell contamination was ruled out by exclusion of HLA-DR⁺ cells (Suppl. Fig. 1A). Gated on total NK cells, we determined the frequency of CD8⁺ cells in a
cross-sectional analysis of 33 untreated and 27 treated HIV-seropositive individuals and 15 uninfected control subjects (Fig. 3A, B). Importantly, the samples from untreated HIV patients were randomly taken and thus represent a variety of clinical stages of HIV immunopathogenesis. We observed substantially lower frequencies of CD8⁺ NK cells in untreated HIV-1 infected individuals compared to healthy controls (Fig. 3B), which was partially restored after one year of ART. To further investigate the effect of antiviral treatment, we performed a follow-up study in previously untreated patients one year after the initiation of ART. Both, the relative numbers as well as the absolute counts of CD8⁺ NK cells were significantly increased in patients after receiving antiviral treatment (Fig. 3C). In summary, we show that HIV-infection leads to a significant decline of CD8⁺ NK cells. Our cross-sectional as well as longitudinal patient data indicate that antiretroviral treatment can restore the number of CD8⁺ NK cells almost to levels comparable to uninfected control subjects.

To further explore the relationship between HIV disease states and the frequency of CD8⁺ NK cells, we investigated the association of CD8⁺ NK cells with CD4⁺ T cell counts and viral loads in untreated HIV-1 seropositive individuals. We observed a modest negative correlation between frequencies of CD8⁺ NK cells with HIV viral loads (r = -0.33, P = 0.01) and a significant, positive correlation with absolute CD4⁺ T cells counts (r = 0.33, P = 0.009; Fig. 3D, E). There was also a significant positive correlation between frequencies of CD8⁺ NK cells and frequencies of CD4⁺ T cells (r = 0.26, P = 0.046; Fig. 3F). Since the ratio of CD4⁺ to CD8⁺ T cells is an important clinical parameter in HIV-caused immune disease, we furthermore performed a correlation analysis between the frequencies of CD8⁺ NK cells and the CD4/CD8 T cell ratio. There was a trend for a positive correlation between the percentages of CD8-expressing cells and the CD4/CD8 T cell ratio although the result did not reach statistical significance (r = 0.1, P = 0.1; Fig. 3G). Altogether, our data indicate a
correlation of CD8\(^+\) NK cells with multiple surrogate markers of HIV-caused immune disease.

Phenotypic characterization of CD8\(^+\) NK cells

The activity of NK cells is regulated by a sophisticated array of germline-encoded activating or inhibiting receptors (2, 4). Having shown that the frequency of CD8\(^+\) NK cells represents a correlate for delayed HIV disease progression also reflected in the correlation with various HIV disease markers, we hypothesized that CD8\(^+\) NK cells possessed a different repertoire of NK cell receptors compared to CD8\(^-\) NK cells. To test our hypothesis, we performed a detailed phenotypic analysis of CD8\(^+\) and CD8\(^-\) NK cells. However, neither in untreated HIV-infected patients (Fig. 4A) nor in uninfected control subjects (Suppl. Fig. 1B) were we able to find significant differences in the percentages of cells expressing CD62L, CD69, CD2, CD94, NKG2D, NKP30, NKP46, NKP80, CD85j, NKG2A and KIR2DL2/DL3.

We also measured PD-1 expression on NK cells. PD-1 expression was detectable on NK cells ex vivo in several subjects but we found no difference in the frequency of cells expressing PD-1 when comparing CD8\(^+\) to CD8\(^-\) NK cells (data not shown).

The absence of notable phenotypic differences when studying NK cell receptors one at a time prompted us to investigate the co-expression patterns of either inhibiting or activating NK cell receptors. To this end, we performed Boolean gating analysis for the inhibiting receptors CD85j, KIR2DL2/DL3 and NKG2A or activating receptors NKG2D, NKP30, NKP46 and NKP80 on either CD8\(^+\) or CD8\(^-\) NK cells. Neither in HIV-infected patients (Fig. 4B, C) nor in uninfected control subjects (data not shown) were we able to find substantial differences in the majority of the co-expression patterns of these receptors in CD8\(^+\) compared to CD8\(^-\) NK cells. Statistically significant differences in frequencies were found for NK cells expressing only NKP30 among activating receptors and only NKG2A among inhibiting receptors, which were both higher among CD8\(^-\) NK cells (Fig. 4B, C).
We and others had previously demonstrated that CD57 expression on NK cells is consistent with a phenotype resembling terminal differentiation (20, 29, 30). To address the differentiation status of these subsets, we thus analyzed the expression of the senescence marker CD57 on CD8⁺ and CD8⁻ NK cells. In HIV-seronegative control subjects CD8⁺ NK cells exhibited marginally higher frequencies of CD57-expressing cells compared to their CD8⁻ counterpart (Suppl. Fig. 1C). We also observed a modest difference in HIV-1 seropositive individuals (Fig. 4D) indicating a moderately more differentiated phenotype in CD8⁺ NK cells. Finally, we determined the frequency of CD56bright, CD56dim or CD56⁻ cells among either CD8⁺ or CD8⁻ NK cells and found almost identical NK cell subset distributions (Fig. 4E). These data thus indicate that apart from a moderately more differentiated phenotype of CD8⁺ NK cells as indicated by higher numbers of CD57-expressing cells, these cells exhibit a remarkably similar phenotype compared to CD8⁻ NK cells.

The CD8⁺ subset exhibits a more functional profile compared to CD8⁻ NK cells

Increasing levels of CD57 expression on NK cells were shown to be associated with an increased expression of perforin and granzyme B (20). Having shown that CD8⁺ NK cells are more likely to express CD57, we thus hypothesized a higher cytolytic potential within this NK cell subpopulation. To test this hypothesis, we assessed the ex vivo intracellular expression of perforin and granzyme B in untreated HIV-seropositive subjects and in our control group. Indeed, higher frequencies of granzyme B- and perforin-expressing cells were detected among CD8⁻ NK cells compared to CD8⁻ cells in HIV-1 infected patients as well as in our cohort of HIV-seronegative blood donors (Fig. 5A, Suppl. Fig. 2A). Since an increase of granzyme B⁺ and perforin⁻ cells is not necessarily reflected in their capacity to degranulate (20), we evaluated the functional capacity of CD8⁺ and CD8⁻ NK cells by performing an intracellular cytokine staining assay. To this end, sorted NK cells were stimulated in the presence of K562 cells as well as IL-12 and IL-15 to achieve a robust activation inducing both, cytokine
production as well as degranulation. We measured the degranulation marker CD107a on the
cell surface and the intracellular expression of IFN-γ and TNF-α. In addition, we were
interested in the ability of NK cells to produce MIP-1β since previous studies indicated a
virus-suppressive role for NK cell-derived CC-chemokines by competitive blocking of the
HIV co-receptor CCR5 (31-33). These experiments were carried out in 20 untreated HIV-
seropositive subjects, which were a subset of the aforementioned patients from the cross-
sectional study, selected on the basis of sample availability as well as viral loads (patients
with low as well as high viremia). For all four functions, there was a moderate, yet
statistically significant increase of responding cells in the CD8⁺ NK cell subpopulation
compared to CD8⁻ cells in untreated HIV-positive individuals (Fig. 5B). In uninfected control
subjects, we observed a similar increase of cells expressing IFN-γ, MIP-1β and CD107a
among CD8⁺ NK cells (Suppl. Fig. 2B).

We further studied the functional capacity of CD8⁺ and CD8⁻ NK cells responses by
performing Boolean analysis of NK cells expressing IFN-γ, CD107a, TNF-α and MIP-1β
after stimulation. Notably, the frequencies of cells exhibiting all four functions were
significantly enhanced in the CD8⁺ NK cell subset compared to CD8⁻ cells in both, untreated
HIV-seropositive subjects as well as uninfected controls (Fig. 5C, D). In a similar vein, the
frequencies of cells performing none of the four functions were higher in the CD8⁻ NK cell
subpopulation compared to CD8⁺ cells (Fig. 5C). Since these data were derived from
untreated HIV-1 seropositive patients with varied viral loads, we divided the patients into
groups with viral loads below or above 10,000 copies/ml. With the exception of one outlier,
polyfunctional cells were not increased in CD8⁺ NK cells compared to CD8⁻ in patients with
viral loads above 10,000 copies/ml. The majority of individuals with enhanced functional
activities of CD8⁺ NK cells were found in HIV-seropositive subjects with viral loads below
10,000 copies/ml (Fig. 5D).
We also sought to determine the proliferative activity of CD8\(^+\) and CD8\(^-\) NK cells \textit{ex vivo}. To this end, we analyzed the intracellular expression of Ki-67, a marker associated with cycling cells. As shown previously (18, 34), a greater number of Ki-67\(^+\) cells were observed in untreated HIV-infected individuals compared to uninfected subjects (Suppl. Fig. 2C). However, we were unable to detect differences in the relative frequencies Ki-67\(^+\) cells in CD8\(^-\) compared to CD8\(^+\) NK cells, both in HIV-infected as well as uninfected subjects (Suppl. Fig. 2C). This finding suggests that the prevalence of cycling cells among CD8\(^+\) NK cells is similar compared to CD8\(^-\) NK cells.

In summary, our data indicate that CD8\(^+\) NK cells are characterized by a greater responsiveness to stimulation with MHC I-devoid target cells and cytokines. These cells therefore exhibit an enhanced functional profile as further shown by the co-expression pattern of IFN-\(\gamma\), CD107a, TNF-\(\alpha\) and MIP-1\(\beta\).
Discussion

Accumulating evidence suggests that human NK cells are of pivotal importance in the host defense against viruses, including HIV (35). Whereas epidemiological and genetic data highlight the importance of NK cell contributions to viral control, to the best of our knowledge direct NK cell correlates with delayed progression to AIDS have not been demonstrated yet.

Here, we performed a retrospective analysis of 117 untreated HIV-patients who had been followed longitudinally at our outpatient clinic. We show that chronically infected, untreated HIV patients with high numbers of CD8⁺CD3⁻ lymphocytes were associated with delayed HIV disease progression, which we defined as a drop of CD4⁺ T cell counts below 200 cells/μl or ART initiation. Upon closer examination, we could show that the majority of these CD8⁺CD3⁻ cells were well-described, peripheral blood NK cells, expressing either CD56 or CD16 or both. Notably, high numbers of bulk NK cells were also associated with a delayed onset of AIDS. We subtracted the absolute numbers and percentages of CD8⁺CD3⁻ lymphocytes from the respective NK cell numbers, which provided an approximation of CD8⁻ NK cells with the caveat of varying numbers of CD56/CD16 double-negative cells within the CD8⁺CD3⁻ lymphocyte population. The resulting analysis indicated that CD8⁺ rather than CD8⁻ NK cells represent a correlate with slower disease progression. Nonetheless, further studies employing a consistent gating strategy to define CD8⁻ and CD8⁺ NK cells and their association with disease progression are warranted.

A drawback in our analysis is that the precise time point of first infection with HIV in all of our subjects remains elusive thus introducing a potential bias mediated by a possible overrepresentation of long-term non-progressors. In our longitudinal study cohort, we identified 7 elite controllers, which exhibited robust viral control throughout the entire study with viral copy numbers undetectable by conventional methods at most time points assessed (data not shown). None of these patients reached our end point (median follow-up time 53.6
months). The estimated frequency of HIV-1 patients with such durable viral control is usually 1 in 300 (36) indicating a possible overrepresentation of elite controllers in our study cohort.

Viral control can be mediated by a number of adaptive immune mechanisms, such as HIV-specific CD8+ T cells, in particular those restricted by protective HIV alleles, including HLA-B*57 or HLA-B*27 (37, 38). In addition, the advent of single-cell antibody cloning techniques has led to the discovery of dozens of highly potent and broadly neutralizing antibodies from HIV-seropositive patients (39, 40). The potential impact of these cellular and humoral immune mechanisms and their protective effects in HIV infection could not be accounted for in our study. Thus, although we took various HIV disease parameters into account in our survival analyses, there are additional known factors, which we were not able to address and which could confound our study.

Nonetheless, our survival data is corroborated by cross-sectional data derived from a subset of these patients and further samples from HIV-infected individuals on ART and uninfected controls. We have recently demonstrated an inverse correlation between the frequency of CCR7-expressing CD56bright NK cells and HIV copy numbers in plasma and a positive correlation with CD4+ T cells counts (18). Here, we demonstrated that the frequency of CD8+ NK cells, similarly to CCR7+CD56bright NK cells, is also negatively correlated with HIV viral load, and positively correlated with absolute and relative CD4+ T cell frequencies. We thus provide evidence for correlations of CD8+ NK cells with multiple HIV-related clinical disease parameters emphasizing the potential usefulness of CD8+ NK cells as a novel NK cell-derived, inverse biomarker for HIV pathogenesis. Similarly to CCR7-expressing CD56bright NK cells, our cross-sectional as well as longitudinal results strongly suggested that the frequencies of CD8+ NK cells could be partially restored by suppressing viral loads by initiation of ART. We furthermore found a modest correlation between CCR7+CD56bright NK cells and CD8+ NK cells (data not shown).
Our observation of CD8\(^+\) NK cell loss in chronic HIV-1 infection is a confirmation of earlier studies reporting a selective depletion of CD16\(^-\)CD8\(^+\) or CD56\(^+\)CD16\(^-\)CD8\(^+\) NK cells in HIV infection (41, 42). However, the mechanism of the selective loss of C8\(^+\) NK cells remains elusive. Although direct infection of CD16\(^-\)CD8\(^+\) NK cells and viral replication in this subset had been suggested (43) it represents an unlikely mechanism to fully explain the dramatic loss of CD8\(^+\) NK cells since the expression of CD4 in conjunction with the HIV coreceptors CCR5 or CXCR4 on NK cells is rather scarce (44). An alternative explanation could be that CD8\(^+\) NK cells are more susceptible to apoptosis. More so than other human peripheral blood lymphocyte subpopulations, CD8\(^+\) NK cells were shown to be particularly sensitive to irradiation of PBMCs and their loss correlated with the dose or \(\gamma\) irradiation (45).

Furthermore, the ligation of CD8 on the cell surface of NK cell clones using either soluble MHC class I molecules or HLA-G was shown to induce apoptosis (46). Although this finding was challenged by data indicating that engagement of CD8\(^+\) on NK cells prevented apoptosis (9), the discrepancy could be explained by the fact that triggering of apoptosis was Fas-dependent, which was present on NK cell clones but absent on freshly isolated NK cells (9). Given that Fas is significantly up-regulated on NK cells in chronic HIV infection (18, 34), ligation of CD8 followed by subsequent Fas-mediated apoptosis could represent a viable hypothesis for the selective loss of CD8\(^+\) NK cells.

Furthermore, our data emphasize that a better understanding of the precise role of CD8 on human NK cells is required. The surface expression of the homodimer CD8\(\alpha\alpha\) on NK cells was observed in rats (47) and pigs (48) but was reported to be absent in mice (49) and thus seems to vary across different mammalian species (50). In rhesus macaques, one of the best-studied non-human primate models, virtually all NK cells express CD8 (28, 51, 52). In contrast, in chimpanzees, which are phylogenetically substantially closer to humans, peripheral blood NK cells were reported to consist of CD8\(^-\) as well as CD8\(^+\) subsets (53). Chimpanzee CD8\(^+\) NK cells apparently expressed more NKp46 and exhibited a more
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Functional profile whereas CD8− cells were described to be functionally anergic cells (53). However, a subsequent study indicated that CD8− NK cells as defined by Rutjens et al. could be contaminated with mDCs, which in turn might account for the phenotypic differences between CD8+ and CD8− cells (54). Thus, the overwhelming majority of peripheral blood NK cells in chimpanzees seem to express CD8, which is in contrast to human NK cells. This could be an indication that CD8− and CD8+ NK cells assume different roles in chimpanzees compared to humans. Several studies addressed the role of CD8+ NK cells during HIV-1 infection in chimpanzees and found no decline of these cells (53, 55, 56). One likely explanation is arguably the observation that immune pathologies caused of HIV-1 infection in humans substantially vary from chimpanzees since HIV-1 infected chimpanzees do not progress to AIDS although a seminal study demonstrated the development of AIDS in wild chimpanzees infected with naturally occurring SIVCPZ (57).

Notably, in our phenotypic analyses we were unable to identify any striking differences between CD8− and CD8+ NK cells when comparing the expression of various activating and inhibiting NK cell receptors one at a time. After performing Boolean gating and analyzing every co-expression permutation of four activating or three inhibiting receptors, we identified a difference in the frequency in NK cells expressing NKp30 in the absence of NKG2D, NKp46 and NKp80 and NK cells expressing NKG2A in the absence of KIR2DL2/DL3 and CD85j. Furthermore, we found a modest increase in the frequency of CD57-expressing cells among CD8+ NK cells. These findings could be an indication that 10-color polychromatic flow cytometry as performed in this study is insufficient for a rigorous delineation of the diversity of NK cell phenotypic states. A recent study employed 37-parameter Time of Flight Mass Cytometry to simultaneously assess the expression of 28 NK cell receptors on human PBMCs (58). Subsequent Boolean gating analysis enabled the authors to study NK cells with unprecedented depth and uncovered tens of thousands of distinct expression patterns of NK cell receptors indicating that the enormous diversity and heterogeneity of human NK cells had...
been vastly underappreciated (58). Thus, it remains a likely possibility that a more detailed in-depth analysis of NK cells at a single cell level could uncover further differences in the co-expression patterns of various NK cell receptors on CD8⁺ and CD8⁻ NK cells, which could help elucidating the functional role of CD8 on human NK cells.

Previous studies reported CD8⁺ NK cells to be more cytolytic compared to CD8⁻ NK cells (9, 59). In accordance with these studies, we identified the CD8⁺ subset to exhibit a more functional profile, which was evident when analyzing single functions as well as co-expression patterns of four functional markers. We were thus able to confirm previously published results and could further extend these observations by providing additional data on IFN-γ, TNF-α and MIP-1β. Interestingly, NK cells derived from HIV elite controllers carrying the KIR3DL1*h/*y in conjunction with HLA-B*57 were shown to be more responsive to K562 cells and, very similarly to CD8⁺ NK cells as shown in our study, exhibited a more polyfunctional profile (13). Similarly to our data, Kayam et al. showed that the functionality of NK cells was impacted by the viral burden in HIV-1 patients although viral loads were not a major determinant for the functional capacity of the NK cells (13). Thus, it will be of great interest to evaluate in future studies how host genetics can shape the phenotypic and functional profile of NK cells, in particular with respect to CD8 expression.

In conclusion, we presented evidence for a role of CD8⁺ NK cells as a novel innate immune correlate for delayed disease progression and as a robust inverse marker for HIV-induced immune disease. Future studies are warranted to address whether the high prevalence of these cells in HIV-infected patients with slower disease progression is merely an epiphenomenon or whether these cells can make an active contribution to viral control as suggested by their increased functionality.
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Author Contributions

F.A. H.S.H and D.M.O. conceived the study and designed the experiments. F.A., H.S.H., I.L., N.B., J.M.E., B.A.B. M.B., and M.Z.K. performed the experiments. F.A., H.S.H., M.J., A.J. and D.M.O. analyzed the data. M.B. provided technical assistance. F.A., H.S.H., D.M.O. and R.E.S. prepared the manuscript. All authors read and commented on the manuscript.

The authors declare no competing financial interest.
References


**Figure legends**

**Figure 1:** High absolute number and high frequencies of CD8^+^CD3^-^ cells are associated with slower HIV-disease progression. Patients were ranked according to their absolute or relative numbers of CD8^+^CD3^-^ cells and subjects belonging to the first tertile (n = 39) were compared to the patients belonging to the second and third tertiles (n = 78). Kaplan-Maier survival analyses were performed to study effects of either high or low absolute and relative baseline numbers of CD8^+^CD3^-^ cells on disease progression. Endpoints were defined as (A, B) ART initiation or drop of CD4^+^ T cell numbers below 200 cells/µl or (C, D) ART initiation only. (E) Survival comparison of patients with either high or low absolute CD8^+^CD3^-^ lymphocytes after censoring patients with ART initiation at CD4^+^ T cells > 359 cell/µl is shown. (F) Survival of patients with high absolute CD8^+^CD3^-^ cell counts (n = 37) were compared to patients with low CD8^+^CD3^-^ cell counts (n = 73) after exclusion of 7 elite controllers.

**Figure 2:** CD8^+^CD3^-^ cells are mostly CD56-expressing NK cells. (A) The gating strategy employed to analyze CD8^+^CD3^-^ cells is shown. (B) Pie charts comparing the composition of CD8^+^CD3^-^ lymphocytes in terms of NK cell subsets in 15 seronegative control and 37 HIV-1 seropositive subjects are shown. (C, D) Kaplan-Maier survival analyses were performed to compare the progression-free survival of untreated HIV-seropositive patients. Subjects were ranked according to their absolute (C) or relative NK cells counts (D). Patients with “high” NK cell numbers belong to the first tertile and “low” belong to the second and third tertiles. (E, F) Kaplan-Maier survival analyses of untreated HIV patients comparing the first tertile to the second and third tertiles based on absolute counts (E) or relative frequencies (F) of CD8^+^CD3^-^ cell-subtracted NK cells.
Figure 3: Frequencies of CD8⁺ NK cells correlate with clinical disease parameters of HIV-1 infection. (A) Representative histogram showing CD8 expression on gated NK cells in healthy controls and in HIV-1 patients. (B) Frequencies of CD8⁺ NK cells were compared in 15 control subjects and in either 45 untreated or 28 treated HIV-infected patients. (C) Analysis of the frequency and absolute counts of CD8⁺ NK cells in 21 HIV-infected patients before and after treatment. Paired student’s t test was performed to determine statistically significant differences. (D) Spearman’s rank correlation analysis of frequency of CD8⁺ NK cells in 60 untreated HIV-infected patients with HIV-viral load and (E) CD4⁺ T cell counts. Spearman’s rank correlation analysis of frequency of CD8⁺ NK cells in 60 untreated HIV-infected subjects with percentages of (F) CD4⁺ T cells or (G) CD4/CD8 T cell ratio. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 4: Phenotypic analyses of CD8⁺ and CD8⁻ NK in untreated HIV-infected individuals. (A) The frequency of CD8⁺ and CD8⁻ NK cells expressing various NK cell receptors was compared. Bars indicate means of 37 untreated HIV-infected individuals. (B, C) Co-expression patterns of (B) inhibiting receptors CD85j, KIR2DL2 and NKG2A and (C) activating receptors on CD8⁺ and CD8⁻ NK cell subsets are shown. Pies and bar graphs indicate the means from all 37 untreated HIV-infected subjects. Statistically significant differences are denoted. (D) The frequency of CD8⁺ and CD8⁻ NK cells expressing CD57 is shown. Horizontal bars show the mean. (E) Frequencies of CD56bright, CD56dim and CD56neg NK cell subsets within CD8⁺ and CD8⁻ NK cells are shown. Bar graph shows the means of data derived from 37 untreated HIV-seropositive patients. *, P < 0.05; **, P < 0.01.

Figure 5: CD8⁺ NK cells display a more functional profile. (A) Summary data comparing the frequencies of CD8⁺ and CD8⁻ NK cells expressing granzyme B or perforin cells in 35 untreated HIV-infected patients. (B) Summary data of frequencies of CD8⁺ and CD8⁻ NK...
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cells expressing IFN-γ, TNF-α, MIP-1β or CD107a from 20 untreated HIV-infected patients after stimulation with K562 cells, IL-12 and IL-15 are shown. Bars show the mean. (C) Co-expression patterns of CD107a, IFN-γ, MIP-1β and TNF-α in CD8+ and CD8- NK cells are shown. Pie charts show the means from 20 untreated HIV-seropositive subjects and 9 uninfected control subjects. Bar graph shows the mean of 20 untreated HIV-infected blood donors. (D) The frequencies of NK cells expressing all four functional markers (IFN-γ, TNF-α, MIP-1β and CD107a) were analyzed in 9 healthy control subjects and untreated HIV-infected patients with either high (>10,000 copy/mL, n=8) or low viral loads (<10,000 copy/mL, n=12).

Table 1: Patient’s characteristics at baseline and end point. All values are provided as medians and the interquartile ranges are indicated in brackets.
Table 1: Patient’s characteristics at baseline and end point. All values are provided as medians and the interquartile ranges are indicated in brackets.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Endpoint</th>
</tr>
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<tbody>
<tr>
<td>Lymphocytes (n/μl)</td>
<td>2,207 (1,917 to 2,653)</td>
<td>1,781 (1,323 to 2,264)</td>
</tr>
<tr>
<td>CD3 T cells (n/μl)</td>
<td>1,759 (1,510 to 2,132)</td>
<td>1,393 (1,082 to 1,823)</td>
</tr>
<tr>
<td>% CD3 T cells of lymphocytes</td>
<td>81 (77 to 85)</td>
<td>81 (76 to 86)</td>
</tr>
<tr>
<td>CD4 T cells (n/μl)</td>
<td>606 (546 to 711)</td>
<td>339 (280 to 505)</td>
</tr>
<tr>
<td>% CD4 T cells of lymphocytes</td>
<td>29 (24 to 34)</td>
<td>23 (18 to 28)</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>0.59 (0.46 to 0.77)</td>
<td>0.4 (0.29 to 0.58)</td>
</tr>
<tr>
<td>Viral load (copies/ml)</td>
<td>13,247 (2,425 to 34,600)</td>
<td>23,623 (5,443 to 95,877)</td>
</tr>
<tr>
<td>CD8+CD3 (n/μl)</td>
<td>64 (49 to 101)</td>
<td>45 (27 to 75)</td>
</tr>
<tr>
<td>% CD8+CD3 of lymphocytes</td>
<td>3 (2 to 4)</td>
<td>3 (2 to 4)</td>
</tr>
<tr>
<td>NK cells (n/μl)</td>
<td>179 (120 to 270)</td>
<td>105 (66 to 174)</td>
</tr>
<tr>
<td>% NK cells of lymphocytes</td>
<td>8 (6 to 11)</td>
<td>7 (4 to 11)</td>
</tr>
</tbody>
</table>
Figure 1

A C
% Progression free
Time (months)

B
% CD8+CD3- High
% CD8+CD3- Low
P = 0.003

D
% CD8+CD3- High
% CD8+CD3- Low
P = 0.012

E
ART > CD4+ 359 censored
P = 0.028

F
EC excluded
P = 0.0015
Figure 2

A. Flow cytometry plot showing CD8+CD3− and CD8−CD3+ cell populations.
B. Pie charts comparing NK cell populations between Control and HIV groups.

N = 15  N = 37

C. Graph showing progression free time for NK cells High vs Low, P = 0.011
D. Graph showing progression free time for % NK cells High vs % NK cells Low, P = 0.14

E. Graph showing progression free time for NK cells – CD8+CD3− High vs Low, P = 0.24
F. Graph showing progression free time for % NK cells – % CD8+CD3− High vs Low, P = 0.46
Figure 3

A

B

C

D

E

F

G

Control HIV

% CD8+ NK cells

Pre-ART Post-ART

P = 0.0079

r = 0.33

Pre-ART Post-ART

P = 0.009

r = 0.26

Pre-ART Post-ART

P = 0.1

r = 0.22

CD4/CD8 T cell ratio

% CD8+ NK cells

Pre-ART Post-ART

P = 0.046

r = 0.2

CD4+ T cells

% CD8+ NK cells

Pre-ART Post-ART

P = 0.004

r = -0.33

Log_10 HIV viral load

CD8

% CD8+ NK cells

Pre-ART Post-ART

P = 0.01

r = 0.33

% CD8+ NK cells

Pre-ART Post-ART

P < 0.0001

P = 0.01

r = -0.33

% CD8+ NK cells

Pre-ART Post-ART

P = 0.0017

r = 0.33

% CD8+ NK cells

Pre-ART Post-ART

P = 0.0017

r = 0.33
Figure 4

A

B

C

D

E

Arc Legend
- CD8
+ CD8

** P = 0.0066

Frequency (%)
Figure 5

A. % Granzyme B* (P = 0.001, P = 0.005)

B. % IFN-γ* (P = 0.018, P = 0.046), % MIP-1β* (P = 0.004, P = 0.038)

C. CD8− vs. CD8+ (HIV vs. Control) Pie Chart Arc Legend

D. % Polyfunctional cells (P = 0.013, P = 0.027)