RESEARCH PAPER

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Analysis of NKp30/NCR3 isoforms in untreated HIV-1-infected patients from the ANRS SEROCO cohort

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Abbreviations: DC, dendritic cell; GIST, gastrointestinal stromal tumor; HD, healthy donor; KIR, killer-cell immunoglobulin-like receptor; MFI, mean fluorescence intensity; NCR, natural cytotoxicity receptors; NK, natural killer; PBMC, peripheral blood mononuclear cell

Natural killer (NK) cells play a prominent role at the intersection between innate and cognate immunity, thus influencing the development of multiple pathological conditions including HIV-1-induced AIDS. Not only NK cells directly kill HIV-1-infected cells, but also control the maturation and/or elimination of dendritic cells (DCs). These functions are regulated by the delicate balance between activating and inhibiting receptors expressed at the NK-cell surface. Among the former, NKp30 has raised significant interest since the alternative splicing of its intracellular domain leads to differential effector functions, dictating the prognosis of patients bearing gastrointestinal sarcoma, and B7-H6 has recently been identified as its main ligand. Since NKp30 is downregulated in $CD56^{-}/CD16^{+}$ NK cells expanded in viremic, chronically infected HIV-1^{+} patients, we decided to investigate the predictive value of NKp30 splice variants for spontaneous disease progression in 89 therapy-naïve HIV-1-infected individuals enrolled in an historical cohort of patients followed since diagnosis (ANRS SEROCO cohort). We found no difference in the representation of NK-cell subsets ($CD56^{bright}$, $CD56^{dim}$, $CD56^{neg}$) in HIV-1-infected patients as compared with healthy subjects. NKp30 downregulation was detected in $CD56^{dim}$ and $CD56^{neg}$ NK-cell subsets, yet this did not convey any prognostic value. None of the NKp30 isoforms did affect disease progression, as measured in terms of time-to-loss of circulating $CD4^{+}$ T cells, time-to-AIDS-defining events and overall survival. NKp30 isoforms do not seem to play a major role in the outcome of HIV-1 infection, but the heterogeneity of the immuno-virological status of patients at enrollment could have to be taken into account.

Introduction

Accumulating evidence suggests an important role for innate immunity in the control of acute HIV-1 infection prior to the establishment of adaptive immune responses, as well as in the subsequent rate of viral replication and disease progression.[1]

A vast array of receptors with either inhibitory or activating functions regulates the interaction between natural killer (NK) cells and other cells. Uninfected and untransformed "self" cells are recognized by inhibitory NK-cell receptors that sense normal HLA Class I molecules expression levels and prevent NK-cell activation.[2] Killer-cell immunoglobulin-like receptors (KIRs) are the main receptors for HLA Class I molecules (i.e., HLA-A, HLA-B, HLA-C and HLA-E).[2] The major NK-cell activating molecules include natural cytotoxicity receptors (NCRs) (i.e., NKp46, NKp30 and NKp44) and NKG2D, which are readily triggered by ligands expressed at the surface of infected and transformed cells.[3] The activating NK-cell receptor NKp30 is involved in both dendritic cells (DC) killing and DC maturation,[4] and appears not only to be critical for tumor-cell recognition[5] but also to influence the prognosis of different infectious diseases.[6–12] The human NKp30-encoding gene (*NCR3*) is transcribed in six different splice variants,[13] among which the most highly expressed are NKp30a, b and c.[14]

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The significance of NK-cell antiviral activity in vivo is indicated by the fact that HIV-1 evolved specific strategies to evade NK-cell responses. Indeed, the viral protein Nef acts on infected cells by selectively downregulating the expression of HLA-A and HLA-B (preventing cells to be recognized and eliminated by T cells), but not of HLA-C and HLA-E (protecting cells from NK-cell cytotoxicity).[15] Nef also induces the downregulation of ligands for the activating NK receptors NKG2D (i.e., MICA, ULBP1 and ULBP2)[16] and NKp44.[17] During chronic infection, HIV-1 mutants are detected that enhance the binding of the inhibitory receptor KIR2DL2 to its ligands, thus avoiding the recognition of infected cells by NK cells.[18] The relative amounts of activating and inhibitory KIRs play a role in the containment of viral replication in HIV-1-infected individuals.[19] Furthermore, NK cells seem to be relevant determinants for the outcome of HIV-1 infection, as the deletion of the gene encoding the NK-cell activating receptor NKG2C is a risk factor for HIV-1 infection,[20] and increased NK-cell activity has been correlated with protection from infection in several cohorts of highly-exposed seronegative subjects.[21]

HIV-1 infection is associated with a functional impairment of NK cells that is evident early after infection and persists during disease progression,[22] leading to alterations of the DC/NK crosstalk.[6,23,24] In viremic HIV-1-infected patients, reduced NK-cell function is associated with low expression of NCRs[25] as well as with the expansion of the "anergic" $CD56^{-}/CD16^{+}$ ($CD56^{neg}$) NK-cell subset,[26] which is characterized by reduced NKp30 expression, decreased cytolytic functions and low cytokine production capacity.[26,27] The exhaustion of NK cells in chronic HIV-1-infected patients leads to altered DC editing, manifesting with an impaired killing of autologous immature DCs (iDCs). In particular, the markedly impaired expression and function of NKp30 among $CD56^{neg}$ NK cells subset largely accounts for the highly defective NK cell-mediated lysis of autologous iDCs.[6] In turn, mature DCs generated from HIV-1 viremic patients are substantially impaired in their ability to induce the proliferation of autologous NK cells, which consequently fail to secrete adequate amounts of interferon γ (IFNγ).[6] On the contrary, HIV-1-infected chimpanzees, which control infection when exposed to human-adapted HIV-1 variants, maintain functionally competent NK cells with high NCR expression during the course of infection,[7] confirming the importance of NCRs in this setting.

We have recently characterized NKp30 isoforms, demonstrating functional differences among the three major NKp30 splice variants: whereas NKp30a-transfected NKL cells (a human NK cell line)[28] block the proliferation of tumor cells harboring the NKp30 ligand B7-H6,[5] exhibit granule exocytosis into the microenvironment and kill tumor cells as well as iDCs, NKL cells that express NKp30b or NKp30c fail to do so (though the former preserve the capacity to respond to B7-H6-harboring cells by secreting T_H1 cytokines).[29] Most interestingly, in a retrospective analysis of 80 patients affected by gastrointestinal stromal tumor (GIST), a neoplasm that expresses NKp30 ligands and is sensitive to NK cell-mediated lysis, a predominant expression of the NKp30c isoform was associated with reduced patient survival, decreased NKp30-dependent tumor necrosis factor α (TNFα) and CD107a release, as well as defective IFNγ and interleukin (IL)-12 secretion in the NK-DC crosstalk, which could be restored by blocking IL-10. In line with this notion, the NKp30 status has been shown to predict the clinical outcome of patients with GIST.[29]

Considering (1) the critical role of NKp30 during NK-dependent DC maturation or killing and the subsequent polarization of immune responses, (2) the alteration of NKp30 and NKp46 expression on NK cells following HIV-1 infection and (3) the different functions of the three major isoforms of NKp30, we sought to determine the potential prognostic impact of the genetically determined NKp30 status on the control of HIV-1 infection in a historical cohort of HIV-1-infected untreated patients, the ANRS SEROCO.

Results

Expression of NKp30 and NKp46 receptors on peripheral blood NK cells. Peripheral blood mononuclear cells (PBMCs) from $HIV\text{-}1^{+}$ patients (n = 89) and healthy donors (HDs) (n = 10) were analyzed by flow cytometry to determine the relative abundance of NK-cell subsets as well as their expression levels of NKp30 and NKp46 (**Fig. 1A**). NK cells were identified as $CD3^{-}$, $TCR\gamma\delta^{-}$, $CD56^{+}$ and/or $CD16^{+}$ cells (**Fig. 1**). Among total NK cells, three subpopulations were defined based on the levels of expression of CD56 and CD16: $CD56^{bright}/CD16^{-}$ ($CD56^{bright}$), $CD56^{dim}/CD16^{+/-}$ ($CD56^{dim}$) and $CD56^{-}/CD16^{+}$ ($CD56^{neg}$). For each of these NK-cell subtypes, the percentage of $NKp30^{+}$ or $NKp46^{+}$ cells was evaluated together with the mean fluorescence intensity (MFI) of NKp30 or NKp46 expression on positive cells (**Fig. 1**).

We found no significant difference in the percentage of NK cells nor in the distribution of NK-cell subsets ($CD56^{bright}$, $CD56^{dim}$, $CD56^{neg}$) between $HIV\text{-}1^{+}$ patients and HDs (**Fig. S1**). In particular, the average percentage of $CD56^{neg}$ cells in HDs was 15.6% (6.0–26.7%) and in HIV^{+} patients 26.9% (4.0–77%). Nevertheless, we observed a decrease in the percentage of NK cells expressing NKp30 in $HIV\text{-}1^{+}$ patients as compared with HDs (p = 0.0011) (**Fig. 1B**), while the expression level of NKp30 on a per cell basis (evaluated by MFI on positive cells) remained stable (**Fig. 1B**). A reduction in the percentage of $NKp30^{+}$ cells was also observed among $CD56^{dim}$ and $CD56^{neg}$ cells of HIV-1-infected subjects compared with HD-derived cells (p = 0.085 and p = 0.0168, respectively) (**Fig. 1B**). Meanwhile, no significant differences were detected in the expression levels of the activating receptor NKp46, regardless of the NK-cell subset considered (**Fig. 1B**). Hence, HIV^{+} individuals exhibit a downregulation of NKp30 expression on peripheral NK cells.

NKp30 expression levels and clinical predictors. To analyze whether a reduced expression of NKp30 was related to transcriptional defects, we isolated total RNA from the PBMCs of 89 $HIV\text{-}1^{+}$ patients and 87 HDs, and quantified the expression levels of the three major NKp30 isoforms (a, b and c) using quantitative reverse transcription-PCR (qRT-PCR) and the ΔΔCt analysis method. The mean ΔCT ($Ct_{NKp30} - Ct_{β2M}$) values for NKp30a, NKp30b and NKp30c in the HD group were 9.69, 7.35 and 8.83, respectively.

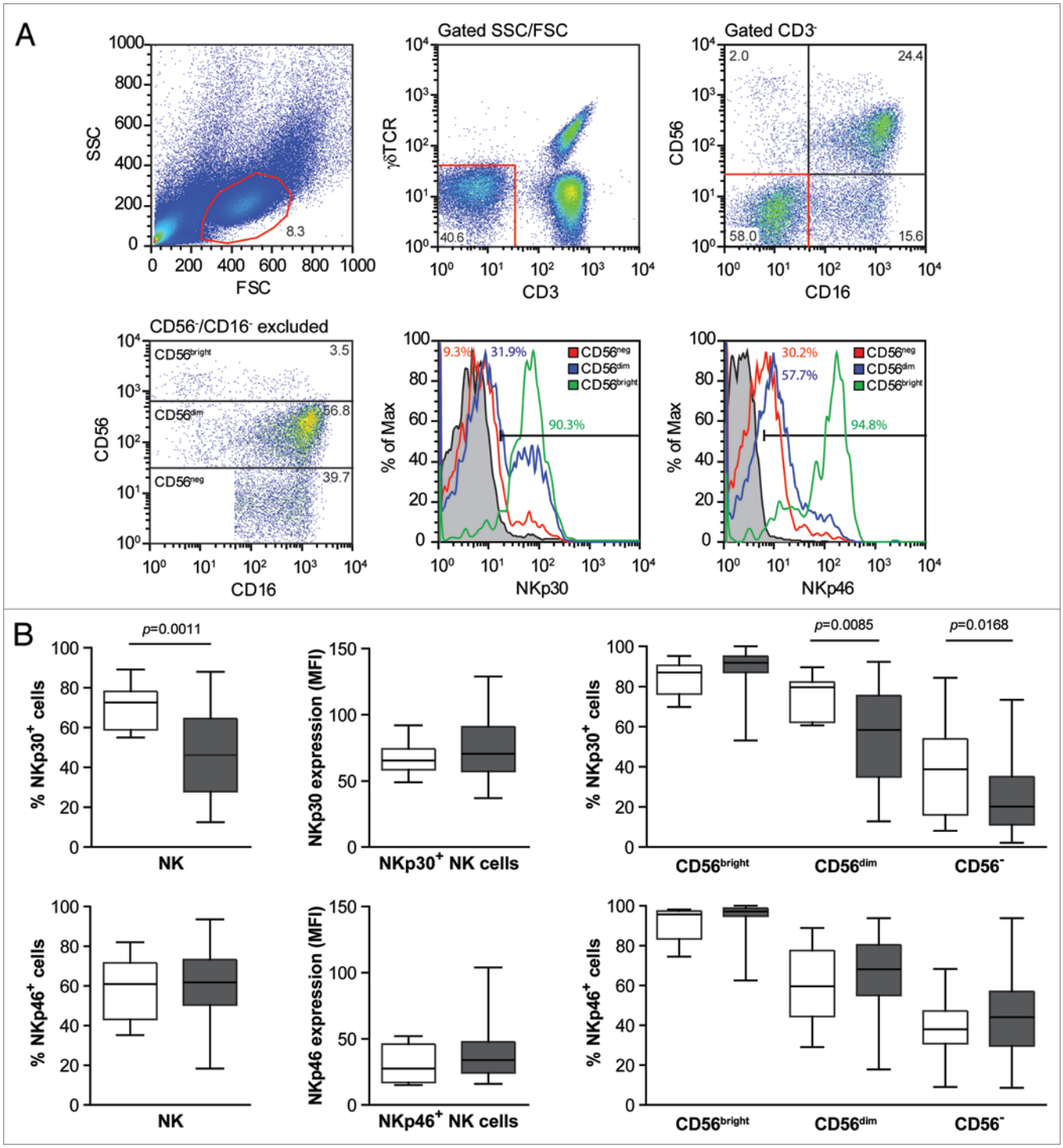

Figure 1. HIV-1+ seroconverters exhibit lower percentage of NK cells expressing NKp30 on their membrane, as compared with healthy donors. (**A and B**) Frozen peripheral blood mononuclear cells (PBMCs) isolated from HIV-1+ patients and healthy donors (HDs) were stained with CD3, CD16, CD56, γδTCR, NKp30 and NKp46-specific antibodies and analyzed by flow cytometry. (**A**) Gating procedure and NKp30 and NKp46 expression among natural killer (NK) cells of the indicated subsets (one representative experiment out of 89 is shown). (**B**) White and gray box plots represent data for HDs (n = 10) and HIV-1+ subjects (n = 74), respectively (middle bars = median values, box plots = 25% and 75% percentiles, whiskers = minimum and maximum values). Statistically significant p values are reported (unpaired, two-tailed Student's t-test).

By means of an unsupervised hierarchical clustering based on log-transformed and median-centered data, HIV-1+ patients were then clustered into three groups reflecting the mRNA expression level of the three NKp30 isoforms, compared with HDs (ΔΔCt cluster): high NKp30 (n = 20, ΔCT NKp30a: 9.66; NKp30b: 7.49; NKp30c: 8.95, comparable to the values of HDs), intermediate

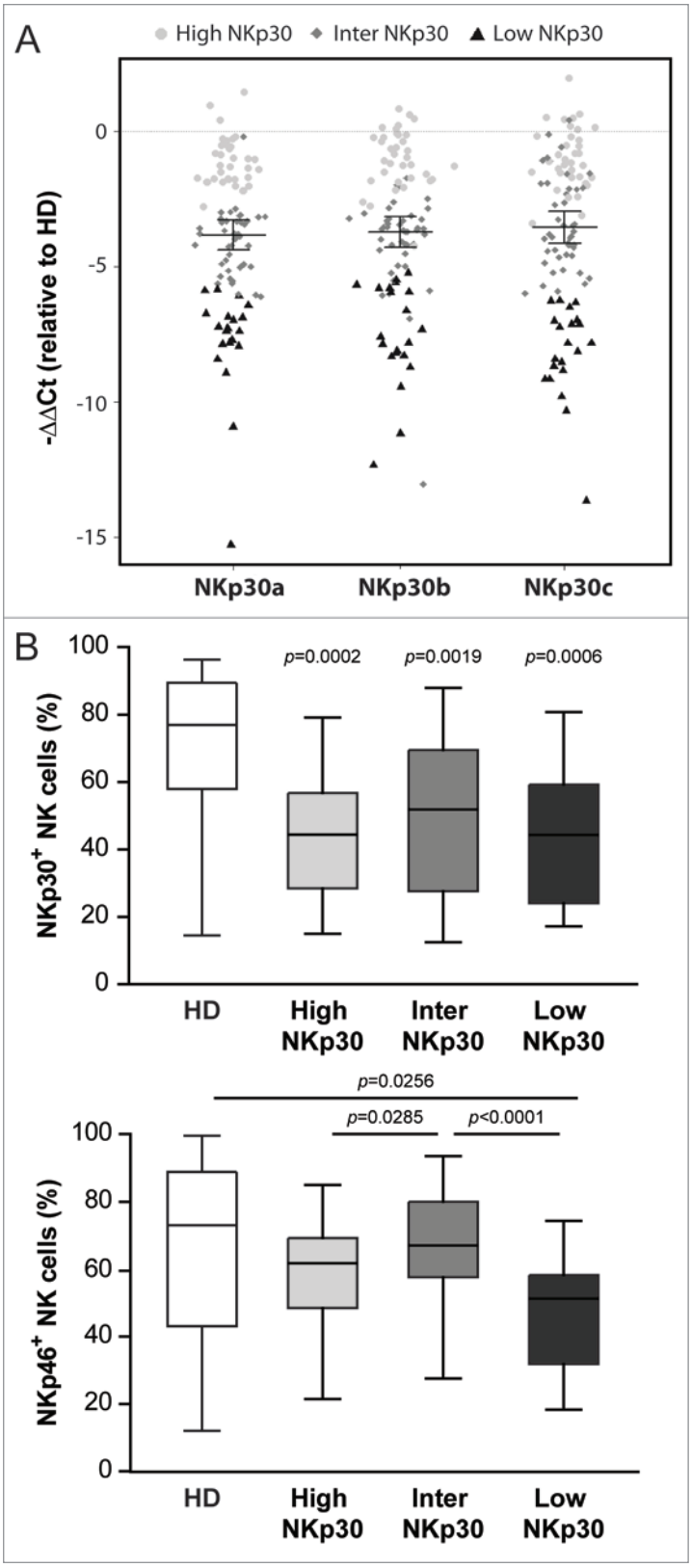

Figure 2. Seroconverters clustered based on *NCR3* mRNA expression levels do not differ in terms of relative abundance of NKp30$^+$ NK cells. (**A and B**) Total RNA was isolated from the peripheral blood mononuclear cells (PBMCs) of HIV-1$^+$ patients and healthy donors (HDs) and quantified by qRT-PCR. (**A**) HIV-1$^+$ patients were clustered into three groups based on *NCR3* expression levels as compared with the HD group (ΔΔCt cluster). The difference between NKp30 levels in HIV$^+$ patients and HDs is reported. (**B**) Percentage of NKp30$^+$ or NKp46$^+$ natural killer (NK) cells for each of the three patient groups as identified by the ΔΔCt cluster are shown. Middle bars = median values, box plots = 25% and 75% percentiles, whiskers = minimum and maximum values. Statistically significant p values are reported (unpaired, two-tailed Student's t-test). In the upper panel, p values refer to the difference between each group of HIV-1$^+$ subjects and HDs.

NKp30 (n = 42, ΔCT NKp30a: 12.36; NKp30b: 10.02; NKp30c: 10.95) and low NKp30 (n = 23, ΔCT NKp30a: 16.20; NKp30b: 13.46; NKp30c: 15.83) (**Fig. 2A; Table 1**). NKp30 mRNA expression level could not be determined in 4 patients.

For each group of patients defined by the ΔΔCt cluster, the percentage of NK cells expressing membrane NKp30 or NKp46 was determined by flow cytometry (**Fig. 2B**). Among the three ΔΔCt cluster groups, the percentages of NKp30$^+$ NK cells were comparable (**Fig. 2B**, upper panel), although significant differences were found in the percentage of NKp46$^+$ NK cells (**Fig. 2B**, lower panel), suggesting that the reduced expression of NKp30 on the surface of NK cells from HIV$^+$ individuals does not result from transcriptional alterations of the three main NKp30 isoforms.

We then addressed whether the mRNA expression levels of *NCR3* may influence the progression of HIV-1 infection. For each of the three ΔΔCt cluster groups, we evaluated the time- to-CD4$^+$ T-cell loss (based on the number of patients whose CD4$^+$ cell count fell below 200 cells/mm^3 at two consecutive visits) (**Fig. 3A**), the time-to-first AIDS-defining illness (**Fig. 3B**) and survival (**Fig. 3C**). We observed no association between *NCR3* mRNA levels in the three ΔΔCt cluster groups and these parameters (p = 0.89, p = 0.93, p = 0.54, for CD4$^+$ T-cell count fall, AIDS and survival, respectively).

Relative NKp30 isoform expression levels and clinical predictors. The levels of expression of the three major NKp30 isoforms were measured by qRT-PCR using RNA extracted from PBMCs, purified total NK cells, CD56^{bright}, CD56^{dim} and CD56^{neg} NK-cell subsets from 10 HIV-1$^+$ patients. The relative expression of the different isoforms compared with each other was calculated using the "ratio" formula: $NKp30_x$ / $NKp30_y$ = 2^(ΔΔCt $NKp30_y$ – ΔΔCt $NKp30_x$). The relative expression of NKp30 isoforms was similar in all cell subsets analyzed (**Fig. S2**). Furthermore, the NKp30 isoform profile was stable over time, as shown by a longitudinal analysis performed in the 10 HIV-1$^+$ patients at two time points with a mean temporal distance of 5.5 y (**Fig. S3**).

Unsupervised hierarchical clustering was subsequently performed on the relative NKp30 isoform expression data from 56 HDs and 89 HIV-1$^+$ patients. The A vs. B, B vs. C and A vs. C distribution on HDs is shown in **Figure S4**. The clustering of HIV$^+$ subjects resulted in the definition of three groups of patients with distinct NKp30 profile (ratio cluster): patients presenting as the most remarkable feature a low expression level of the c isoform (Low C, n = 40), a high expression level of the b isoform (High B, n = 15) and a high expression level of

Table 1. Patients' characteristics and ΔΔCt cluster

Characteristics	Total (n = 89)	#NKp30^{High} (n = 20)	#NKp30^{Int} (n = 42)	#NKp30^{Low} (n = 23)	Statistics	
Sex*					p^1	
Men	67 (75%)	15 (75%)	31 (74%)	17 (74%)	**1**	
Women	22 (25%)	5 (25%)	11 (26%)	6 (26%)		
Mode of transmission*						
Men from Men	31 (69%)	13 (65%)	27 (64%)	17 (74%)		
Men from Women	6 (7%)	2 (10%)	4 (10%)	0 (0%)	**0. 69**	
Women from Men	22 (25%)	5 (25%)	11 (26%)	6 (26%)		
Time (in months) from infection to **					p^2	
Inclusion	6.2 [1.3–43.5]	5.4 [2.7–41.2]	8.9 [1.3–43.5]	6.1 [2.1–10.1]	**0.08**	
NKp30 study	38.3 [3.6–44.4]	40.0 [8.5–44.0]	28.0 [3.6–44.4]	41.8 [17.2–44.4]	**0.008**	
***Biological markers** at inclusion*					p^2	p^3
CD4$^+$ cells	556 [72–1330]	458.5 [268–1260]	542 [72–1193]	616 [224–1330]	**0.05**	**0.09**[a]
Log(HIV-1 RNA)	4.1 [2.4–5.6]	4.3 [3.5–5.6]	4.1 [3.1–5.3]	3.9 [2.4–5.5]	**0.18**	**0.18**[a]
Log(HIV-1 DNA)	2.8 [1.7–4.0]	2.8 [1.8–3.6]	2.7 [1.7–3.7]	2.9 [1.7–4.0]	**0.57**	**0.60**[a]
***Biological markers** at* **NKp30 status assessment**						
CD4$^+$ cells	478 [19–1193]	479 [244–797]	490 [72–1193]	456 [19–917]	**0.60**	**0.62**[b]
Log(HIV-1 RNA)	4.0 [2.4–5.6]	3.7 [3.0–5.5]	4.0 [3.0–5.1]	4.0 [2.7–5.6]	**0.99**	**0.99**[b]
Log(HIV-1 DNA)	2.9 [1.6–5.2]	3.0 [2.0–5.2]	2.9 [1.9–3.7]	3.0 [1.6–4.0]	**0.75**	**0.45**[b]

#ΔΔCt cluster groups; *number (%); **median [min-max]; [1]Fisher's exact test; [2]Kruskal-Wallis rank sum test; [3]Linear regression adjusted for: [a]mean time from contamination to inclusion in the study, [b]mean time from contamination to NKp30 status assessment.

the c isoform (High C n = 28) (**Fig. 4A; Table 2**). The NKp30 isoform profile could not be determined in 6 patients.

In each of these three groups, we assessed the percentage of NKp30$^+$ or NKp46$^+$ NK cells (by flow cytometry). The three groups of patients showed a decrease in the percentage of NKp30$^+$ NK cells as compared with HDs ($p < 0.0001$, $p = 0.0064$ and $p = 0.0031$ for the Low C, High B and High C groups, respectively) but no difference was detected in the percentage of NKp30$^+$ or NKp46$^+$ NK cells between the three ratio cluster groups (**Fig. 4B**).

We finally searched for a potential influence of NKp30 isoform expression profile on disease evolution. The Kaplan-Meier curves shown in **Figure 5** illustrate that there is no association between the NKp30 isoform profile and time-to-CD4+ T-cell loss ($p = 0.58$), time-to-clinical AIDS ($p = 0.64$) or survival ($p = 0.59$).

Frequency of CD56^{neg} NK cells and prognostic factors. As mentioned above, we found no significant expansion of the CD56^{neg} NK-cell subset in HIV-1$^+$ patients as compared with HDs (**Fig. S1**). Nevertheless, we evaluated whether the percentage of CD56^{neg} NK cells would be correlated with the CD4$^+$ cell count, plasma viral load or cell-associated proviral DNA at the time of our study. No association was found between the proportion of CD56^{neg} NK cells and these parameters (**Fig. 6**) among the 89 patients included in the study (on average 6 mo post-infection), with a median serum viral load of 12,589 HIV-1 RNA copies/mL and a CD4$^+$ cell count of 556 cells/mm^3 (**Table 1**).

Discussion

The aim of our study was to assess the potential role of NKp30-related parameters (NKp30 surface expression, NKp30 transcriptional levels, NKp30 isoforms) on the progression of HIV-1 infection. The finding that the preferential expression of the immunosuppressive NKp30c isoform is associated with poor prognosis in GIST patients[29] prompted us to perform a retrospective analysis of 89 HIV-1-infected individuals from the ANRS CO 02 SEROCO-HEMOCO cohort. This cohort included patients enrolled early after HIV seroconversion and followed from 1988 to 1995, before the introduction of highly active antiretroviral therapy (HAART). Thus, this cohort offered an extended follow-up of untreated patients, allowing us to determine the influence of the NKp30 isoform profile on spontaneous disease progression. We found that neither NKp30 expression levels nor the NKp30 isoform profile correlates with the virological and clinical parameters analyzed in this cohort of patients for whom the natural history of HIV-1 infection is available. Nevertheless, we noticed that—at the time of inclusion in the cohort—individuals belonging to the Low B group (exhibiting a reduced expression of NKp30b) were characterized by a significantly higher CD4$^+$ T-cell count and a lower viral load as compared with the other groups of patients (**Table 2**). This might suggest an early effect of the NKp30 profile on the loss of CD4$^+$ cells, which may become irrelevant with the progression of disease. Further studies are needed to test this hypothesis on a more homogeneous cohort of acutely infected patients.

Comparative flow cytometry analyses of NK-cell subsets on frozen PBMCs from HIV-1$^+$ patients and HDs showed no difference in the percentage of NK cells among PBMCs nor in the proportion of CD56^{bright}, CD56^{dim} and CD56^{neg} cells among NK cells (**Fig. S1**). This is in contrast with previous observations by Mavilio et al., who reported that in viremic patients with chronic

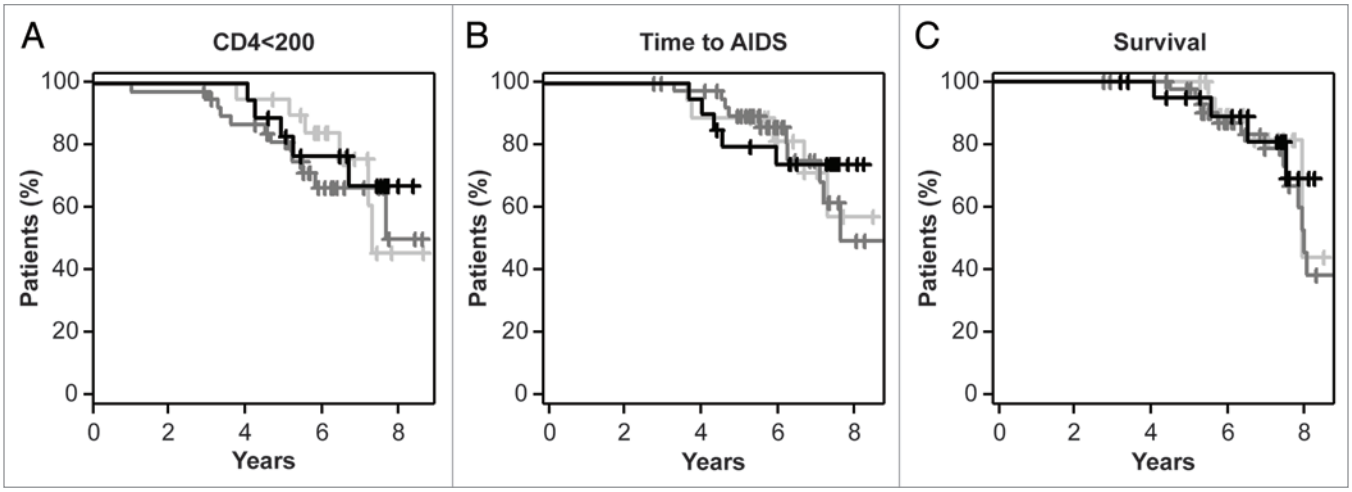

Figure 3. *NCR3* mRNA expression levels neither correlate with the loss of CD4⁺ T cells nor constitute a prognostic factor for time-to-AIDS or overall survival. For each of the three groups of patients as identified by the ΔΔCt cluster (light gray = High NKp30, dark gray = Inter NKp30, black = Low NKp30), Kaplan-Meier curves for time-to-loss of CD4⁺ T cells (< 200/mm³) time-to-first AIDS-defining event and survival are shown.

HIV-1 infection, the proportion of $CD56^{dim}$ NK cells is sharply decreased and the $CD56^{neg}$ NK cells subpopulation is expanded as compared with HDs.[30] This apparent discrepancy may be due to the differences in disease stage, i.e., recent infection vs. chronic late infection, across the two groups of patients studied, suggesting that alterations in the representation of NK-cell subsets require several years of infection to occur.

In HIV-1 infected individuals, we observed a decrease in the percentage of NK cells expressing NKp30, affecting both $CD56^{dim}$ and $CD56^{neg}$ NK cells (**Fig. 1B**). In line with this observation, Mavilio et al. reported that viremic, but not aviremic, chronically infected patients exhibit a significant downregulation of NCRs, including NKp30 and NKp46.[30] In contrast, we observed no difference in the expression of NKp46 between HIV-1⁺ patients and HDs (**Fig. 1B**), suggesting that alterations in NKp46 expression appears later after primary infection. We did not find any association between the percentage of NKp30⁺ NK cells in HIV⁺ patients and the clinical parameters that we evaluated (loss of CD4⁺ T cells, time-to-AIDS-defining event or patient survival) (data not shown).

We subsequently quantified *NCR3* mRNA from the PBMCs of both seroconverters and HDs, normalizing NKp30 expression levels to those of the housekeeping gene β2 microglobulin (*B2M*). The unsupervised hierarchical clustering of *NCR3* mRNA expression data (ΔΔCt clustering) allowed us to classify patients into three groups expressing high, intermediate and low levels of NKp30 (**Fig. 2A**). The High NKp30 group showed NKp30 levels comparable to those observed among HDs (**Fig. 2A**). Interestingly, no correlation between NKp30 mRNA levels (ΔΔCt cluster groups) and surface NKp30 expression levels (assessed by flow cytometry) was found, in terms of both percentage of NKp30⁺ cells (**Fig. 2B**) and MFI on NKp30⁺ cells (data not shown), perhaps suggesting a consistent degree of post-transcriptional regulation of NKp30.

We then addressed the question as to whether the NKp30 isoform profile may influence the progression of HIV-1 infection. We performed qRT-PCR using primers specific for each of the three major NKp30 isoforms (NKp30a, NKp30b and NKp30c) and their relative expression level was calculated for all patients. These expression levels were comparable whether evaluated on the RNA from PBMCs or from purified NK cells, $CD56^{bright}$, $CD56^{dim}$ or $CD56^{neg}$ cells (**Fig. S2**). NKp30 isoform profiles in HIV-1⁺ subjects were found to be stable over time (**Fig. S3**), similar to what has previously been shown for GIST patients,[29] allowing us to analyze a single time point per patient. The unsupervised hierarchical clustering of the relative expression levels of the three isoforms (ratio clustering) resulted in the classification of patients into three groups: Low C, High B and High C (bearing low levels of the c isoform or high levels of the b or c isoforms, respectively) (**Fig. 4A**). These three groups of patients did not differ in terms of percentage of NKp30⁺ NK cells (**Fig. 4B**), nor in terms of NKp30 expression level on NKp30⁺ cells (data not shown). Therefore, our analysis of the influence of the NKp30 isoform profile on HIV-1 disease progression is unlikely to be biased by differences in surface expression levels of NKp30, yet suggest no prognostic significance for this parameters (at least in our cohort).

We have previously reported that the activation of NK cells bearing different NKp30 isoforms results in different functional outcomes. The predominant expression of the immunosuppressive NKp30c isoform has indeed been associated with reduced survival of GIST patients, correlating with defective IFNγ, TNFα and IL-12 production in the NK-DC crosstalk, which could be restored by blocking IL-10.[29] In spite of the important role played by NK cells during both acute and chronic HIV-1 infection,[22] the crucial function of NKp30 in NK-cell activity,[6,25] and the multiple effects exerted by IL-10 during HIV-1 infection,[31] we were not able to detect in our cohort of 89 recently seroconverted HIV-1⁺ patients any association between the *NCR3* mRNA expression levels or NKp30 isoform profiles and the clinical parameters that we evaluated, i.e., the loss of CD4⁺ T cells, the time-to-clinical AIDS and survival (**Figs. 3 and 5**).

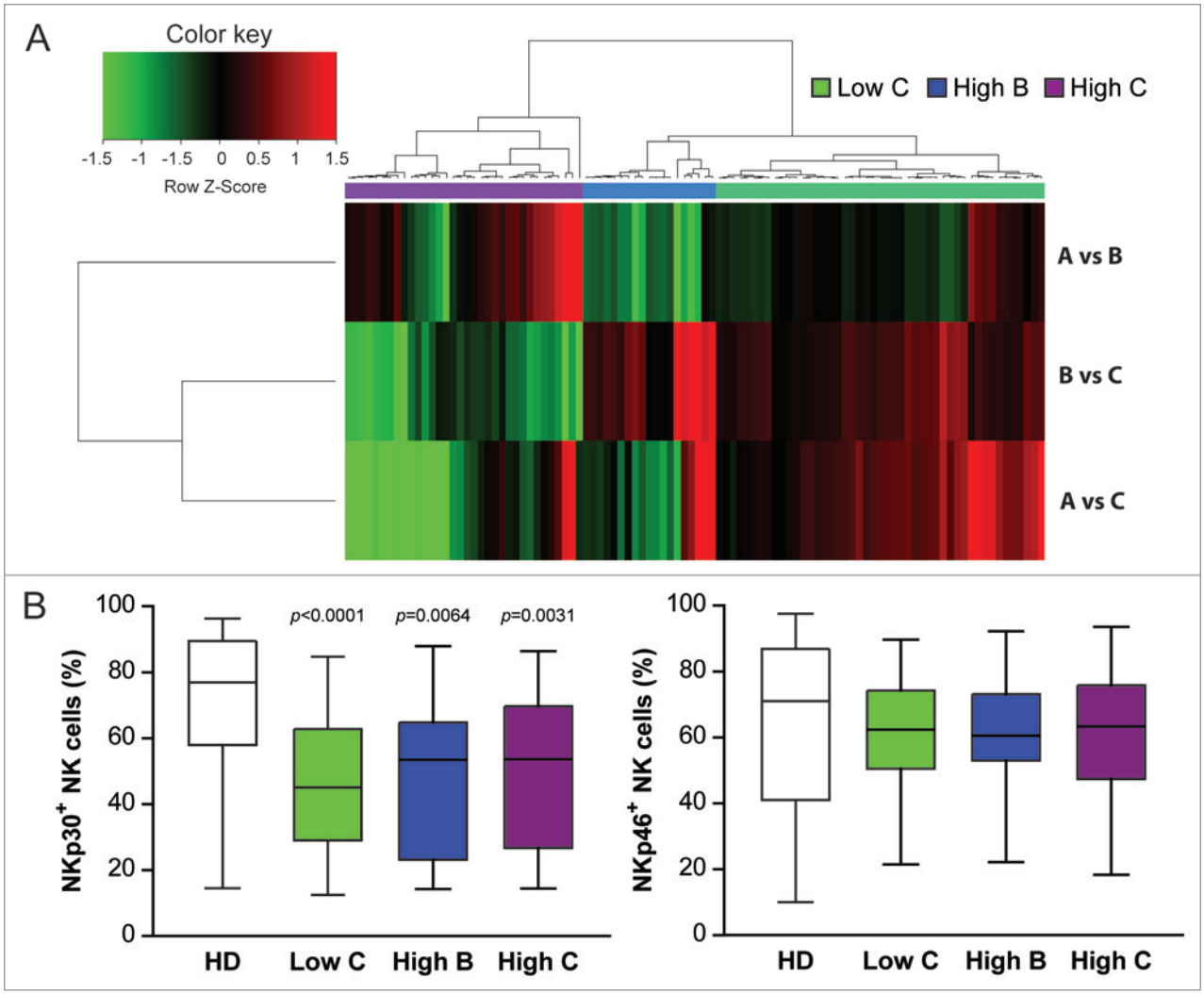

Figure 4. Patients clustered based on their NKp30 isoform profile do not differ in terms of percentage of NKp30$^+$ NK cells. (**A and B**) Total RNA was isolated from the peripheral blood mononuclear cells (PBMCs) of HIV-1$^+$ patients and healthy donors (HDs) and quantified by qRT-PCR. (**A**) HIV-1$^+$ patients were clustered in three groups based on the relative expression levels of the three major NKp30 isoforms (ratio cluster). (**B**) Percentage of NKp30$^+$ or NKp46$^+$ natural killer (NK) cells for each of the three ratio cluster groups. Boxes = 25% and 75% percentiles, middle bars = median values; whiskers = minimum and maximum values. Statistically significant p values are reported (unpaired, two-tailed Student's t-test). In the left panel, p values refer to the difference between each of the three groups of HIV-1$^+$ subjects and HD.

Finally, we did not find any correlation between the percentage of CD56^{neg} NK cells and CD4$^+$ T-cell count, plasma viral load or proviral DNA levels (**Fig. 6**), in contrast with a previous report showing an association between the expansion of the CD56^{neg} subpopulation and viremia.[26] However, our study involves patients at an earlier stage of infection, leading us to speculate that alterations in the NK-cell subsets distribution, notably the expansion of the CD56^{neg} subpopulation, are linked to persistent viral replication and hence constitute a late consequence of immune dysfunction.

Altogether, our observations do not support any correlation between NKp30 status and the clinical outcome of recently infected HIV-1$^+$ patients that were left untreated for more than 3 y. However, we must acknowledge some potential limitations that might have undermined our study. First, although this cohort allowed us to follow the natural evolution of HIV-1 infection, the number of samples per patient was restricted. Second, given the general heterogeneity of HIV-1 infected patients, a greater number of patients may be needed to detect a correlation between NKp30 status and clinical outcome. Finally, due to the limited number of patient analyzed, we could not establish whether the differences among ratio cluster groups in CD4$^+$ T-cell count and viremia at the time of inclusion (**Table 2**) have a biological meaning or reflect biases that may have compromised our analysis. It is time to reevaluate the influence of NKp30 status on the evolution of HIV-1 infection in the setting of primary infection or in long-term non-progressors.

Materials and Methods

Study population. Frozen PBMCs were obtained from patients enrolled in the ANRS CO 02 SEROCO-HEMOCO cohort,

Table 2. Patients' characteristics and ratio cluster

Characteristics	#Low C (n = 40)	#Low B (n = 15)	#High C (n = 28)	Statistics	
Sex*				p^1	
Men	30 (75%)	11 (73%)	22 (79%)	**0.89**	
Women	10 (25%)	4 (27%)	6 (21%)		
Mode of transmission*					
Men from Men	27 (68%)	11 (73%)	19 (68%)		
Men from Women	3 (8%)	0 (0%)	3 (11%)	**0.87**	
Women from Men	10 (25%)	4 (27%)	6 (21%)		
Time (in months) from infection to **				p^2	
Inclusion	6.0 [1.3–43.5]	6.8 [1.8–35.5]	6.6 [1.3–36.5]	**0.72**	
NKp30 study	38.7 [4.7–44.3]	35.5 [10–44]	39.2 [3.6–44.4]	**0.75**	
Biological markers** at inclusion				p^2	p^3
$CD4^+$ cells	485.5 [202–987]	874 [415–1330]	542 [72–1193]	**< 0.01**	**< 0.01[a]**
Log(HIV-1 RNA)	4.2 [3.4–5.6]	3.5 [2.4–4.9]	4.1 [2.9–5.5]	**0.06**	**0.02[a]**
Log(HIV-1 DNA)	3 [1.8–4]	2.6 [1.7–3.2]	2.7 [1.7–3.5]	**0.17**	**0.16[a]**
Biological markers** at NKp30 status assessment					
$CD4^+$ cells	457.5 [19–987]	578 [305–940]	488 [72–1193]	**0.16**	**0.09[b]**
Log(HIV-1 RNA)	4 [3–5.6]	3.7 [3.1–5]	4 [2.7–5.4]	**0.62**	**0.33[b]**
Log(HIV-1 DNA)	3.2 [1.6–5.2]	2.6 [1.7–3.5]	2.9 [1.9–3.7]	**0.14**	**0.10[b]**

#Ratio cluster groups; *number (%); ** median [min-max]; [1]Fisher's exact test; [2]Kruskal-Wallis rank sum test; [3]Linear regression adjusted for: [a]mean time from contamination to inclusion in the study, [b]mean time from contamination to NKp30 status assessment.

which includes individuals with a recent seroconversion or recent HIV-1 diagnosis enrolled from 1988 to 1995.[32] Among these patients, we selected 89 individuals for whom frozen cells were available, who did not present hepatitis B virus or hepatitis C virus co-infection, who were not intravenous drugs users, hemophiliac, or pregnant, had neither autoimmune diseases nor malignancies within the 5 y preceding their enrolment, had no concomitant or previous treatment with interferon and other cytokines, steroids or other immunomodulators. The characteristics of these patients are reported in **Table 1**. Ten HDs served as controls for immunological parameters. A written informed consent was obtained from patients, in line with the guidelines formulated by local ethical committees.

qRT-PCR. The levels of expression of the three major NKp30 isoforms were measured by qRT-PCR and normalized to the level of expression of the housekeeping gene β-2-microglobulin (*B2M*), as previously described.[29] Total cellular RNA was isolated, by means of the RNeasy Mini kit (Qiagen, 74106), from frozen PBMCs, purified NK cells (isolated from PBMCs by magnetic sorting using the EasySep Human NK Cell Enrichment Kit, from Stem Cell, 19055) or purified NK-cell subpopulations (isolated from total NK cells using the a FACSAria cell sorter, from BD Biosciences). cDNA was synthesized from total RNA using the SuperScript™ III Reverse Transcriptase (Invitrogen, 18080-044) and random primers (Promega, C1181), according to the manufacturer's instructions. The following primers and probes (Applied Biosystems) were used for qRT-PCR: NKp30-EC (Fwd): 5'-TTT CCT CCA TGA CCA CCA GG-3'; NKp30-EX4I (Rev): 5'-TTC CCA TGT GAC AGT GGC ATT-3'; NKp30-EX4II (Rev): 5'-CGG AGA GAG TAG ATT TGG CAT ATT-3'; NKP30-EX4III (Rev): 5'-GGA CCT TTC CAG GTC AGA CAT T-3'; NKp30-Probe (6-FAM/TAMRA): 5'-TGG TGG AGA AAG AAC ATC CTC AGC TAG GG-3'; B2M-F (Fwd): 5'- GAT GAG TAT GCC TGC CGT GT-3'; B2M-R (Rev): 5'-AAT TCA TCC AAT CCA AAT GCG-3'; B2M-Probe (6-FAM/TAMRA): 5'-AAC CAT GTG ACT TTG TCA CAG CCC AA-3'. First-strand cDNA was amplified using TaqMan Gene Expression Master Mix (Applied Biosystems, 4369016) and NKp30 or B2M primers (10 μM) and probes (5 μM) in a final volume of 25 μL. One initial incubation at 50°C for 2 min was followed by one cycle of denaturation (95°C for 10 min) and 45 cycles of amplification (95°C for 15 sec and 60°C for 1 min). qRT-PCR was performed in a StepOnePlus System (Applied Biosystems), samples were amplified in triplicate and the qRT-PCR data were analyzed using the $2^{-\Delta Ct}$ method.

Unsupervised hierarchical clustering. The level of expression of NKp30 isoforms in HIV-1^+ patients compared with HDs was determined using the ΔΔCt method: $-\Delta\Delta CT = -[(\text{HIV-1}_{NKp30} - \text{HIV-1}_{B2M}) - (\text{HD}_{NKp30} - \text{HD}_{B2M})]$. The level of expression of the distinct NKp30 isoforms compared with each other in each patient (ratio) was determined using the following formula: $NKp30_x / NKp30_y = 2\text{^}(\Delta\Delta Ct\, NKp30_y - \Delta\Delta Ct\, NKp30_x)$. Unsupervised hierarchical clustering was applied to log-transformed and median-centered data using the Cluster and TreeView programs (average linkage clustering using Pearson's centered correlation as similarity metric). Two clusters were created: the first one based on the different levels of expression of NKp30 in HIV-1^+ patients compared with HDs (ΔΔCt cluster)

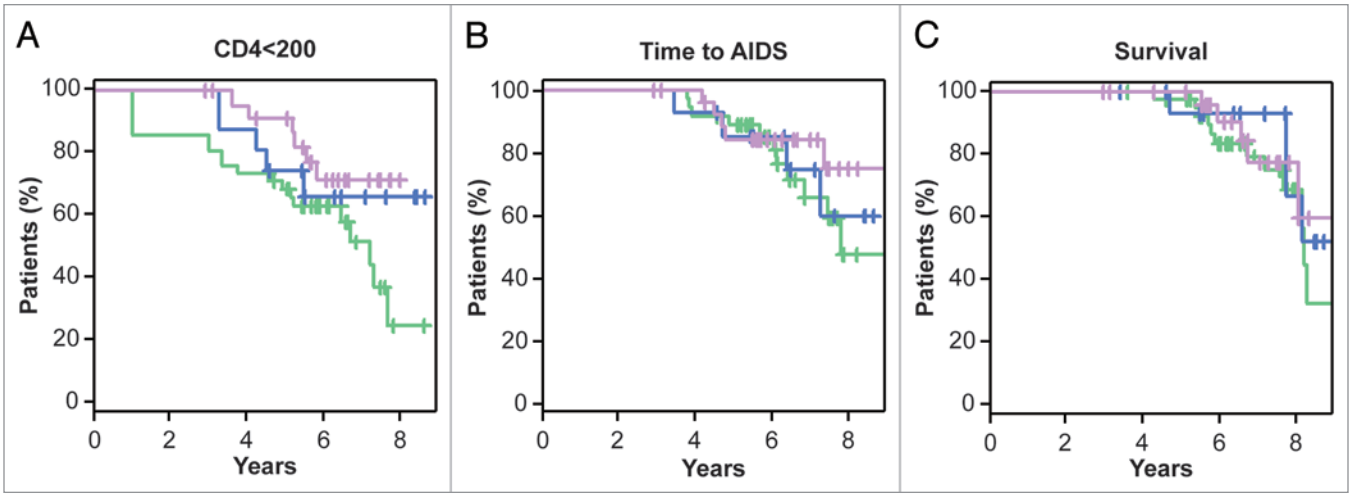

Figure 5. NKp30 isoform profiles do not correlate with the loss of CD4⁺ T cells and are not a prognostic factor for time-to-AIDS or survival. For each of the three groups of patients as identified by the ratio cluster (green = Low C, blue = High B, violet = High C), Kaplan-Meier curves for time-to-loss of CD4⁺ T cells (< 200/mm³), time-to-first AIDS-defining event and survival are shown.

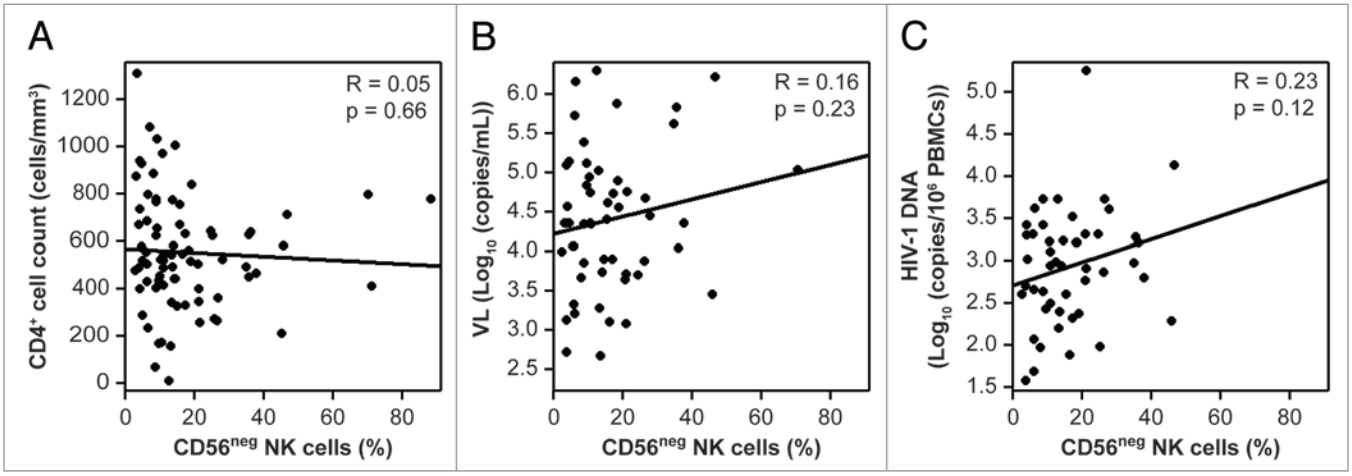

Figure 6. Lack of correlation between the percentages of CD56^{neg} NK cells and clinical parameters. (**A and C**) Percentage of CD56^{neg} cells among natural killer (NK) cells as a function of CD4⁺ T-cell counts (**A**), viral load (**B**) and proviral DNA levels (**C**) (Spearman correlation).

and the second based on the levels of expression of each NKp30 isoform as compared with the others in HIV-1⁺ patients (ratio cluster).

Flow cytometry. The following mouse anti-human fluorescent monoclonal antibodies were used: CD3-APC-Cya7 (Beckman Coulter, A94680), CD16-Pacific Blue (Beckman Coulter, A82792), CD56-PE-Cy7a (Beckman Coulter, A21692), γδTCR-FITC (Beckman Coulter), NKp30-PE (clone AF29-4D12) (Miltenyi, 130-092-483), NKp46-APC (Miltenyi, 130-092-609). In addition, the dead-cell removal reagent LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Invitrogen, L34959) was employed. Frozen cells were thawed, washed and stained with the abovementioned reagents for 15 min at 4°C, washed and fixed with 1% PFA. Cells were acquired on a FACSAria cell sorter immediately following staining and analyses were performed using the FlowJo software (Tree Star).

Statistical analyses. The Fisher's exact test and the non-parametric Kruskal-Wallis rank sum test were used for the comparison of different groups. Survival curves were plotted according to the Kaplan-Meier method. A Cox model was employed to take into account time from infection to NKp30 status assessment (left-entry model) and was used to compare the survival according to NKp30 status. All analyses were performed with the R package version 2.14.2.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be downloaded here:
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