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

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 recognition5 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 cytolysis).5 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 cross-talk.6,23,24 In viremic HIV-1-infected patients, reduced NK-cell function is associated with low expression of NCRs25 as well as with the expansion of the “anergic” CD56dim/CD16− (CD56neg) 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 CD56neg 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 NKp30α-transfected NKL cells (a human NK cell line)28 block the proliferation of tumor cells harboring the NKp30 ligand B7-H6,7 exhibit granule exocytosis into the microenvironment and kill tumor cells as well as iDCs, NKL cells that express NKp30β or NKp30c fail to do so (though the former preserve the capacity to respond to B7-H6-harboring cells by secreting TNFα 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-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−, 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: CD56hi/CD16− (CD56hi), CD56dim/CD16+ (CD56dm) and CD56−/CD16+ (CD56neg). 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 (CD56hi, CD56dm, CD56neg) between HIV-1+ patients and HDs (Fig. S1). In particular, the average percentage of CD56hi 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-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 CD56dm and CD56neg 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 down-regulation 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 untreated patients, the ANRS SEROCO.
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
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/mm³ 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, CD56bright, CD56dim and CD56neg 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: NKp30x / NKp30y = 2^(-ΔΔCt NKp30y − ΔΔCt NKp30x). 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 the a isoform (High A, n = 11).
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 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 significantly higher CD4+ T-cell count and a lower viral load as compared with the other groups of patients) had a significantly lower percentage of NKp30+ NK cells compared to the other groups (p = 0.0031 for the Low C, High B and High C groups, respectively). This is in contrast with previous observations by Mavilio et al., who reported that in viremic patients with chronic...
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, 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 parameter (at least in our cohort).

We subsequently quantified NCR3 mRNA from the PBMCs of both seroconverters and HDs, normalizing NKp30 expression levels to those of the housekeeping gene \(\beta\)-2 microglobulin (B2M). The unsupervised hierarchical clustering of NCR3 mRNA expression data (\(\Delta\Delta\)Ct 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 (\(\Delta\Delta\)Ct 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, 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 parameter (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 IFN\(\gamma\), TNF\(\alpha\) 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).
Finally, we did not find any correlation between the percentage of CD56\textsuperscript{neg} NK cells and CD4\textsuperscript{+} 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\textsuperscript{neg} subpopulation and viremia.\textsuperscript{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\textsuperscript{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\textsuperscript{+} 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\textsuperscript{+} 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,
which includes individuals with a recent seroconversion or recent HIV-1 diagnosis enrolled from 1988 to 1995. 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 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. Total cellular RNA was isolated, by means of the RNasy 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 GAC 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−ΔΔCt method.

Unsupervised hierarchical clustering. The level of expression of NKp30 isoforms in HIV-1+ patients compared with HDs was determined using the ΔΔCt method: −ΔΔCT = −[(HIV-1NKp30 − HIV-1B2M) − (HDNKp30 − HDB2M)]. The level of expression of the distinct NKp30 isoforms compared with eachotherineachpatient(ratio)wasdeterminedusingthefollowing formula: NKp30i / NKp30j = 2^(ΔΔCtNKp30i − ΔΔCtNKp30j). 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).
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: www.landesbioscience.com/journals/onco/article/23472/

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