The Journal of Immunology

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J Immunol 2007; 179:3362-3370; ;
doi: 10.4049/jimmunol.179.5.3362
http://www.jimmunol.org/content/179/5/3362

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Natural Killer Cells in Perinatally HIV-1-Infected Children Exhibit Less Degranulation Compared to HIV-1-Exposed Uninfected Children and Their Expression of KIR2DL3, NKG2C, and NKp46 Correlates with Disease Severity[1]

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NK cells play an integral role in the innate immune response by targeting virally infected and transformed cells with direct killing and providing help to adaptive responses through cytokine secretion. Whereas recent studies have focused on NK cells in HIV-1-infected adults, the role of NK cells in perinatally HIV-1-infected children is less studied. Using multiparametric flow cytometric analysis, we assessed the number, phenotype, and function of NK cell subsets in the peripheral blood of perinatally HIV-1-infected children on highly active antiretroviral therapy and compared them to perinatally exposed but uninfected children. We observed an increased frequency of NK cells expressing inhibitory killer Ig-like receptors in infected children. This difference existed despite comparable levels of total NK cells and NK cell subpopulations between the two groups. Additionally, NK cell subsets from infected children expressed, with and without stimulation, significantly lower levels of the degranulation marker CD107, which correlates with NK cell cytotoxicity. Lastly, increased expression of KIR2DL3, NKG2C, and NKp46 on NK cells correlated with decreased $CD4^+$ T-lymphocyte percentage, an indicator of disease severity in HIV-1- infected children. Taken together, these results show that HIV-1-infected children retain a large population of cytotoxically dysfunctional NK cells relative to perinatally exposed uninfected children. This reduced function appears concurrently with distinct NK cell surface receptor expression and is associated with a loss of $CD4^+$ T cells. This finding suggests that NK cells may have an important role in HIV-1 disease pathogenesis in HIV-1-infected children. ***The Journal of Immunology,*** **2007, 179: 3362–3370.**

Natural killer cells are an integral component of innate immunity that may act directly on target cells by cell-mediated cytotoxicity as well as by the secretion of cytokines and chemokines. They constitute a major lymphocyte population distinct from B and T cells and, in humans, are usually defined as $CD3^-CD56^+$ lymphocytes (1). NK cells can interact with other immune cells, including dendritic cells, and can reciprocally activate one another during an immune response (2). In addition, through their production of cytokines or through cognate cell-cell interaction, NK cells may be able to communicate directly with T cells to enhance adaptive immune responses (3).

NK cells have the capacity to respond to virus-infected and transformed cells that have diminished expression of MHC class I on their cell surface, a process referred to as the "missing-self hypothesis" (4). In humans, the predominant family of inhibitory receptors for MHC class I is the killer cell Ig-like receptors (KIRs)[3] (5, 6). Another family of receptors includes NKG2A and NKG2C, which are C-type lectin-like receptors expressed on the surface of NK cells as heterodimers with CD94. Both heterodimers bind to HLA-E at a partly distinct set of residues. CD94/NKG2A is inhibitory, whereas CD94/NKG2C is an activating complex (7). NK cells also express another activating receptor, NKG2D, that binds to ligands that are up-regulated during viral infection, transformation, and cellular stress, e.g., MIC and ULBPs (8–10). This interaction subsequently leads to NK cell activation. CD161 (NKR-P1A) is also a C-type lectin receptor expressed on NK cells and has an inhibitory function (11, 12). A distinct family of receptors is the natural cytotoxicity receptor (NCR) family that includes NKp30, NKp44, and NKp46, which are expressed on NK cells and thought to play an activating role when they bind to as yet undefined ligands on target cells (13, 14). Unlike NKp30 and NKp46, NKp44 is only expressed on activated NK cells (15, 16).

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Received for publication February 28, 2007. Accepted for publication June 18, 2007.

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[1] Support for this work was provided by the National Institutes of Health (AI064520 and AI060379), the Swedish Research Council, the Swedish Foundation for Strategic Research, the University of California San Francisco Dean's Fellowship, the Alpha Omega Alpha Medical Honors Society Research Fellowship, the American Pediatric Society, and the University of California San Francisco AIDS Research Institute (ARI). L.L.L. is an American Cancer Society Research Professor.

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[3] Abbreviations used in this paper: KIR, killer cell Ig-like receptor; EU, exposed uninfected; FSC, forward scatter; HCMV, human CMV; NCR, natural cytotoxicity receptor.

The absolute number of total lymphocytes declines progressively from the neonatal period through adulthood (17). However, the absolute number of NK cells has been shown to be low in neonates (especially preterm neonates), peaks later in infancy, and eventually recedes to levels in adulthood similar to those of neonates (18–20). Likewise, NK cytotoxic activity correlates directly with gestational age and is diminished in the neonatal period as compared with children and adults (20, 21). The sequence of neonatal NK cells recognizing, binding, killing, and then detaching from a target cell to attack another (i.e., recycling) is depressed (20, 22). This dysfunction or immaturity of neonatal NK cells may reduce the capacity of neonates to respond to viral infections (20, 23–25). The decreased function of cord blood and neonatal NK cells may be partly due to an undefined suppressive mechanism (19). A more recent study suggests that $CD4^+CD25^+$ T regulatory cells could decrease NK cell production of IFN-γ and perforin, most likely through a direct cell-to-cell interaction (26). The phenotype of NK cells derived from cord blood is different from that seen in adults, most notably in the lack of CD57 expression found on many adult $CD56^{dim}$ NK cells (19, 27, 28). Adult $CD57^+$ NK cells are thought to represent senescent cells that have proliferated in vivo (20, 29, 30). However, age-specific phenotypic changes in NK cell populations in children have not been evaluated in detail.

Several studies have suggested a number of potential roles for NK cells in HIV-1 infection. A small subset of NK cells express the CD4 and the HIV-1 coreceptors CCR5 and CXCR4, rendering them potential targets for HIV-1 infection and possibly contributing to the HIV-1 reservoir (31). Other reports also indirectly indicate an important role for NK cells in helping suppress HIV-1 viremia in acute HIV-1 infection and potentially also in the prevention of HIV-1 transmission. For instance, NK cell cytolytic activity in viremic HIV infection is known to be impaired and is associated with a reduced surface expression on NK cells of NCRs (NKp46, NKp30, and NKp44) (32). In addition, it is known that NKp44 is totally absent from the surface of fresh NK cells and is only expressed after they have been activated, and the expression of a putative ligand for NKp44 on $CD4^+$ T cells has been shown to be induced by gp41, rendering the HIV-1-infected $CD4^+$ T cells a target for NK cell cytotoxicity (16). Moreover, epidemiological studies have shown that adult individuals with the KIR3DS1 gene and at least one HLA allele from the public Bw4 epitope bearing an isoleucine at position 80 ($HLA\text{-}Bw4^{Ile80}$) may have slower progression to AIDS (4, 33). Importantly, other reports could not confirm the same effect (34, 35). However, the study by Gaudieri et al. still showed associations between both HLA and the KIR genotype and the outcome of HIV-1 infection (35). Moreover, recent studies have shown that KIR and HLA interactions can affect the risk of HIV-1 transmission and specifically that KIR3DS1 expression triggers NK cell activation (36, 37).

The number and function of NK cells in chronically HIV-1-infected adult subjects is diminished. Specifically, NK cells in viremic, chronically HIV-1-infected individuals have decreased natural cytotoxicity (as measured by the killing of the prototypic NK cell target K562 cell line) (38–42) and decreased Ab-dependent cellular cytotoxicity (ADCC) (41, 43–45). However, a study by Alter et al. has shown that even though viremic HIV-1 infection is associated with a reduction in NK cell numbers and a perturbation of NK cell subsets, the overall NK cell activity is increased (46). In addition, a recent report has shown that in HIV-1-infected viremic patients a subset of NK cells lacking the expression of CD56 ($CD56^-$ NK cells) has an altered expression of NKp30, a NCR, which leads to highly defective NK cell-mediated lysis of autologous dendritic cells (47).

Little is known about NK cell function during the early stages of HIV-1 infection before the development of a strong adaptive immune response. As a first line of defense, NK cells may be particularly important in containing initial viremia and determining the viral load set point. This is especially important in neonates, as their PBMCs have been found to be significantly less active, compared with adults, in Ab-dependent cellular cytotoxicity killing of HIV-1-infected cells (48). Unlike the HIV-1-specific CTL response in young infants, which has been shown to be variable, the physiological role of neonatal NK cells during HIV-1 infection remains unclear (49). Nevertheless, neonatal NK cells have been shown to inhibit HIV-1 replication in vitro, largely by the release of chemokines (50). A recent study demonstrated a partial reconstitution of NK cells after HIV-1 viral suppression in children; however, greater depletion of some NK cell subsets was found to be associated with a clinical history of a decreasing percentage of $CD4^+$ T cells regardless of viral load (51).

In this study, we used multiparametric flow cytometry to evaluate the frequency, phenotype, and function of NK cells in perinatally HIV-1-infected children and compared them to uninfected children born to HIV-1-infected mothers, defined as exposed uninfected (EU) children. We measured the expression of 11 inhibitory and activating receptors on NK cells in addition to intracellular cytokine production at baseline and after stimulation. We also used the CD107 surface expression assay as a marker of the degranulation of NK cells (46, 52, 53). Our results show that whereas both groups had similar absolute NK cell numbers, the NK cells from the HIV-1-infected children degranulated to a lesser extent and a larger fraction expressed KIR2DL1 and KIR3DL1 compared with those of EU children. Interestingly, expression of several NK cell receptors correlated strongly with a decreased percentage of $CD4^+$ T cells.

Materials and Methods

Study subjects and samples

In this study we report data on 39 children. Eleven subjects were HIV-1 perinatally exposed but uninfected, and 28 were perinatally HIV-1-infected. All of these patients were documented to have been infected through mother to child transmission around the time of birth (perinatally) and have been followed in the pediatric HIV clinic at Jacobi Medical Center, Bronx, NY since birth. The race and ethnic background of this cohort of patients is reflective of the population of the Bronx, NY, where the residents are predominately African-American (35.6%) and Hispanic (40.4%) (2000 census) (Table I). The children and/or their guardians provided informed consent in accordance with the institutional review board-approved protocol at the Jacobi Medical Center and at the University of California, San Francisco, CA. As in most studies involving children the recruitment of healthy unexposed controls was difficult, especially given the need to enroll control subjects from the same geographic area (Bronx, NY) and ethnic groups (Hispanic, African-American) for the comparison to be valid. We indeed attempted, with the assistance of our clinical colleagues, to recruit healthy unexposed control subjects into this study. This, however, proved to be logistically difficult, possibly reflecting the basic healthcare challenges that indigent populations who are served by the Jacobi Medical Center face.

Blood (8–10 ml) from each subject was collected by venipuncture in EDTA-containing tubes. The freshly drawn blood samples were shipped overnight from the Bronx, NY to San Francisco, CA. Within 24 h after collection and using Ficoll-Paque Plus density gradient centrifugation (Amersham Biosciences), an average of $8–12 \times 10^6$ PBMCs were isolated from each blood sample and cryopreserved until the time the experiments were performed. Absolute total lymphocyte count, absolute $CD4^+$ T cell count and percentage, HIV-1 plasma viral load (determined by an Amplicor HIV-1 monitor, Roche Diagnostic Systems), and clinical data, including antiretroviral drug therapy history and adherence assessments, were recorded for samples from the HIV-1-infected patients. Data on human CMV (HCMV) serostatus was available for 26 of the 28 HIV-1-infected patients, with 19 patients being HCMV seropositive and seven being HCMV seronegative.

Table I. *Study population*

	Subjects	Age (years)	Sex		Race[a]			Viral Load (HIV RNA copies/ml)	CD4 (%)
			M	F	H	NHB	HB		
EU	11	5.5 (1.6–13.7)	6	5	4	7		NA[b]	NA[b]
HIV$^+$	28	8.2 (2.1–19)	15	13	13	14	1	16 Viremic, 145,989 (2,773–750,000) 12 Aviremic, <400	28.7 (1–50)[c]

[a] H, Hispanic; NHB, non-Hispanic Black; HB, Hispanic Black.
[b] NA, Not applicable.
[c] Data on 27 subjects.

In addition, the 28 HIV-1-infected patients were divided into groups according to the number of HIV-1 RNA copies per milliliter in their serum. The first group consisted of 12 viremic patients (>10,000 HIV-1 RNA copies/ml), and the second group consisted of 12 aviremic patients (<400 HIV-1 RNA copies/ml). The remaining four patients had ≥400 and ≤10,000 HIV-1 RNA copies/ml and were not included in the comparison between viremic and aviremic groups. Viremic subjects had significantly lower absolute numbers of CD4$^+$ T cells ($p = 0.0455$) compared with aviremic subjects. The number of HIV-1 RNA copies/ml serum were measured at more than two time points as part of the HIV-1-infected patients' routine clinical follow-up and were available to us along with the treatment history for at least the 1 year before the study date. All of the 12 aviremic patients had <400 HIV-1 RNA copies/ml during that past year. In addition, all of the HIV-1-infected patients included in this study have been infected since birth, are antiretroviral therapy experienced, and have a wide variation in their viral drug resistance as they all were diagnosed and started on therapy in infancy. The participants were chosen based on being on stable highly active antiretroviral therapy for the year before the time of the study. Even though the treatment regimens were variable they all consisted of a combination of nucleoside reverse-transcriptase inhibitors, most commonly stavudine and lamivudine, with a nonnucleoside reverse transcriptase inhibitor, most commonly efavirenz or either one or a combination of two protease inhibitors, most commonly including nelfinavir. However, the degree of adherence to the prescribed therapy is hard to assess in this population of patients.

Measurement of NK cell frequency, number, and receptor expression

Cryopreserved specimens were thawed and used for measurements of NK cell frequency and receptor expression. The absolute number of NK cells was calculated based on the total number of lymphocytes and the frequency of NK cells as determined by flow cytometry. The freshly thawed cells were washed with PBS supplemented with 1% BSA and 2 mM EDTA (FACS Buffer) before staining. Cells (5×10^5) were incubated with purified human IgG (100 μg/ml) to block nonspecific binding. Samples were divided equally across four different staining panels. Each flow cytometry panel consisted of eight Abs; five of them were common to all of the panels and were used to identify the NK cell population, and three remaining Abs were unique to each panel and used for studying receptor and perforin expression. The common Abs were energy-coupled dye-conjugated anti-CD3 (Beckman Coulter), Alexa Fluor 700-conjugated anti-CD4, allophycocyanin-Cy7-conjugated anti-CD14 and anti-CD19, Pacific Blue-conjugated anti-CD16, and PE-Cy7-conjugated anti-CD56 (BD Biosciences). In addition, each panel contained three different Abs as follows: panel 1, FITC-conjugated anti-KIR3DL1 (clone DX9) (BD Biosciences), PE-conjugated anti-KIR2DL2/3/DS2 (clone DX27) (BD Biosciences), and allophycocyanin-conjugated anti-KIR2DL3 (R&D Systems); panel 2, FITC-conjugated anti-CD94 (BD Biosciences), PE-conjugated anti-NKG2C (R&D Systems), and allophycocyanin-conjugated anti-NKG2A (R&D Systems); panel 3, FITC-conjugated anti-CD57 (BD Biosciences), PE-conjugated anti-KIR2DL1 (R&D Systems), and allophycocyanin-conjugated anti-CD161 (BD Biosciences); panel 4, FITC-conjugated anti-perforin (eBioscience), PE-conjugated anti-NKp44 (Beckman Coulter), and allophycocyanin-conjugated anti-NKp46 (BD Biosciences). Intracellular perforin staining was done after permeabilization of the cells as described in the functional assay.

Cell culture and functional assays

Frozen PBMCs were thawed, washed with RPMI 1640 medium supplemented with 15% FBS, and plated at 5.0×10^5 cells/well in 96-well tissue culture plates. To measure NK cell function, PBMCs were cultured in medium alone, stimulated with 100 ng/ml human rIL-12 (Peprotech) and with 100 μl/ml HIV-1 *gag/nef* pooled peptides obtained from the National Institutes of Health AIDS Research and Reference Program (Rockville, MD) or with the MHC^{null} K562 cell line (American Type Culture Collection) at an E:T ratio of 5:1. The PBMCs cultured in medium only were taken as a measure of "spontaneous" NK cell function. Briefly, thawed PBMCs in 100 μl of medium were cultured at 5×10^6 cells/ml in 96-well plates with the respective stimuli and FITC-conjugated anti-CD107 mAb for 18 h. CD107 expression on the cell surface is a measurement of the degranulation of intracellular vesicles by lymphocytes, including NK cells (46, 53). Anti-CD107 was added at the beginning of the experiment to capture any CD107 expressed on the surface of the NK cell as it is degranulating. During the last 6 h of culture, monensin and brefeldin A were added to block *trans*-Golgi transport and allow the intracellular accumulation of cytokines. The cells were then harvested, washed in FACS buffer, and prepared for Ab staining. Stimulated cells were stained for cell surface Ags (CD3, CD4, CD14, CD19, CD56, and CD16) as described above, and stained with the DNA intercalator ethidium monoazide to exclude dead cells. Cells were fixed in 2% paraformaldehyde and permeabilized with FACS-Perm buffer (BD Pharmingen). Permeabilized cells were then stained for the intracellular cytokines IFN-γ and TNF-α by using allophycocyanin-conjugated anti-IFN-γ and PE-conjugated anti-TNF-α (BD Biosciences), respectively.

Flow cytometry analysis

All stained cells were fixed with 2% paraformaldehyde and analyzed by flow cytometry using a four-laser LSR-II instrument modified from the standard configuration by the addition of a 159-mW green (532 nm) diode laser and the upgrade of the blue and red lasers to 100 and 25 mW, respectively (BD Biosciences). Anti-mouse IgG-coated beads were stained with each fluorochrome separately and used for software-based compensation. Fluorescence-minus-one samples were prepared for each fluorochrome to facilitate gating (54–56).

Data analysis was conducted using the flow cytometric analysis software FlowJo 8.1.1 (Tree Star). For the gating strategy, doublets were excluded based on forward scatter (FSC) height and FSC area. A broad PBMC gate was then defined based on FSC height and side light scatter (Fig. 1*A*). Monocytes and B cells were excluded based on CD14 and CD19 gating, respectively (Fig. 1*B*). CD3$^+$CD4$^+$, CD3$^+$CD4$^-$, and CD3$^-$ cells were gated from the CD14$^-$CD19$^-$ lymphocyte population (Fig. 1*C*). NK cells were identified within the CD3$^-$ gate based on the expression of CD16 and CD56 (Fig. 1*D*). NK cells were then subdivided into CD56^{bright}, CD56^{dim}, and CD56$^-$ (Fig. 1*E*) populations and subsequently analyzed for the expression of other NK cell-specific markers.

Statistical analysis

Statistical analysis was performed by GraphPad Prism statistical software (GraphPad Software). The key outcomes were NK cell percentage and number as well as the percentage and number of NK cells expressing individual markers. The NK cell response was expressed as the percentage and absolute number of NK cells expressing one particular or a combination of cytokines. The nonparametric Mann-Whitney *U* test was used to compare between-group distributions. In addition, the Spearman correlation was used to assess the relationship between the different parameters. The statistical significance threshold was set at $p < 0.05$.

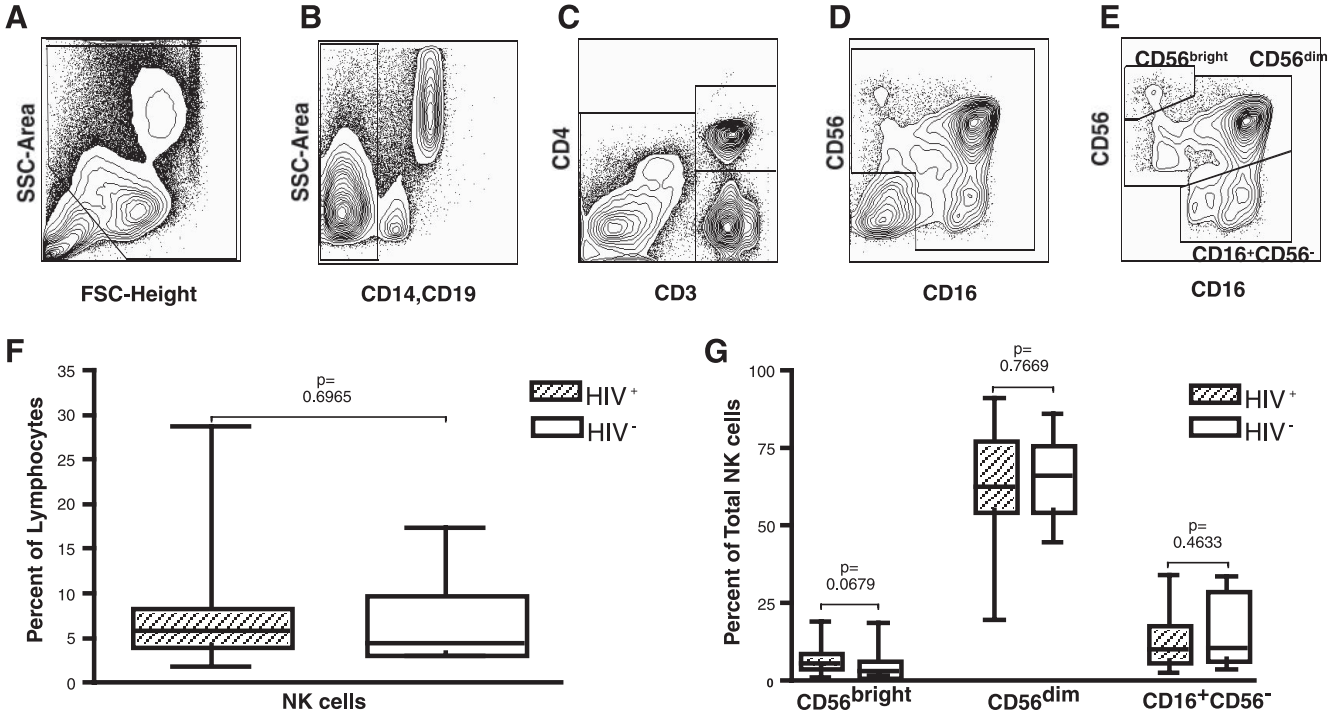

FIGURE 1. Phenotypic analysis of NK cells by nine-color flow cytometry and comparison of NK cell numbers and percentages between HIV-1-infected and HIV-1-EU children. PBMCs were stained directly after thawing with a group of common Abs to identify NK cells in addition to different Abs in each staining panel. *A*, Cells were gated on FSC-height and FSC-area to eliminate doublets followed by side scatter (SSC) and FSC gating to define PBMCs. *B*, CD14 and CD19 staining were used to exclude monocytes and B cells, respectively. *C*, CD3 and CD4 staining of $CD14^-CD19^-$ lymphocytes was used to define $CD3^-$, $CD3^+CD4^+$, and $CD3^+CD4^-$ (which would contain $CD8^+$ T cells). *D*, CD56 and CD16 were used to identify NK cells within the $CD3^-$ population in *C*. *E*, NK cells are divided into subpopulations of $CD56^{bright}$, $CD56^{dim}$, and $CD56^-$. *F* and *G*, NK cell population and subpopulation comparison between HIV-1-infected (HIV^+) and EU (HIV^-) children. The two graphs show no statistically significant difference in the percentages of NK cell population and subpopulations.

Results

Comparable number, phenotype, and function of NK cells in viremic and aviremic HIV-1-infected children

As described in *Materials and Methods*, the 28 HIV-1-infected patients were divided into viremic and aviremic groups depending on the number of HIV-1 RNA copies per milliliter in their serum. When the viremic and aviremic groups were compared there was no significant difference in the number, frequency, phenotype, or function of NK cells (data not shown).

Comparable absolute number and percentage of NK cells in HIV-1-infected and HIV-1-EU children

The number and percentages of total NK cells (Fig. 1*F*) and their subsets ($CD56^{dim}$, $CD56^{bright}$, and $CD56^-$) (Fig. 1*G*) were not different between the perinatally HIV-1-infected children and the HIV-1-EU children even when the $CD4^+$ T cells were gated out of the $CD16^+CD56^-$ population.

Increased frequency of NK cells expressing KIR2DL1 and KIR3DL1 in HIV-1-infected children compared with EU subjects

We compared the frequency of NK cells expressing different activating or inhibitory receptors between HIV-1-infected (HIV^+) and HIV-1-EU (HIV^-) subjects. We found that a higher percentage of NK cells expressed the inhibitory receptors KIR3DL1 (DX9) (Fig. 2; $p = 0.0476$) and KIR2DL1 (Fig. 2; $p = 0.0052$) in the HIV-1-infected group as compared with the EU group. A higher level of NK cells from HIV-1-infected subjects expressed additional surface receptors (DX27, KIR2DL3, CD94, NKG2C, NKG2A, CD57, and CD161); however, the difference did not reach statistical significance (Fig. 2).

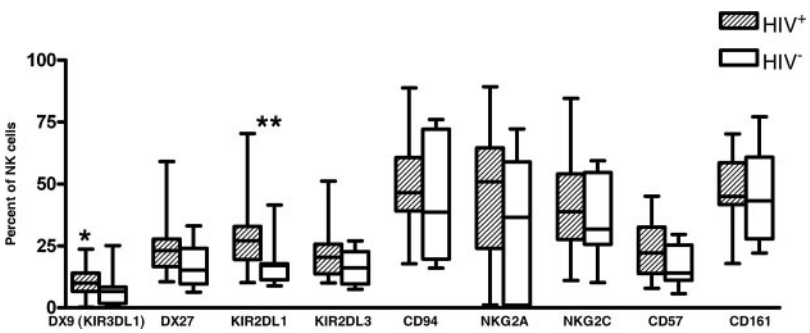

FIGURE 2. Differences between NK subsets in HIV-1-infected (HIV^+) and HIV-1-EU (HIV^-) subjects. Box and whiskers graphs represent the median percent with the interquartile range of NK cells expressing various markers. *, $p < 0.05$; **, $p < 0.01$.

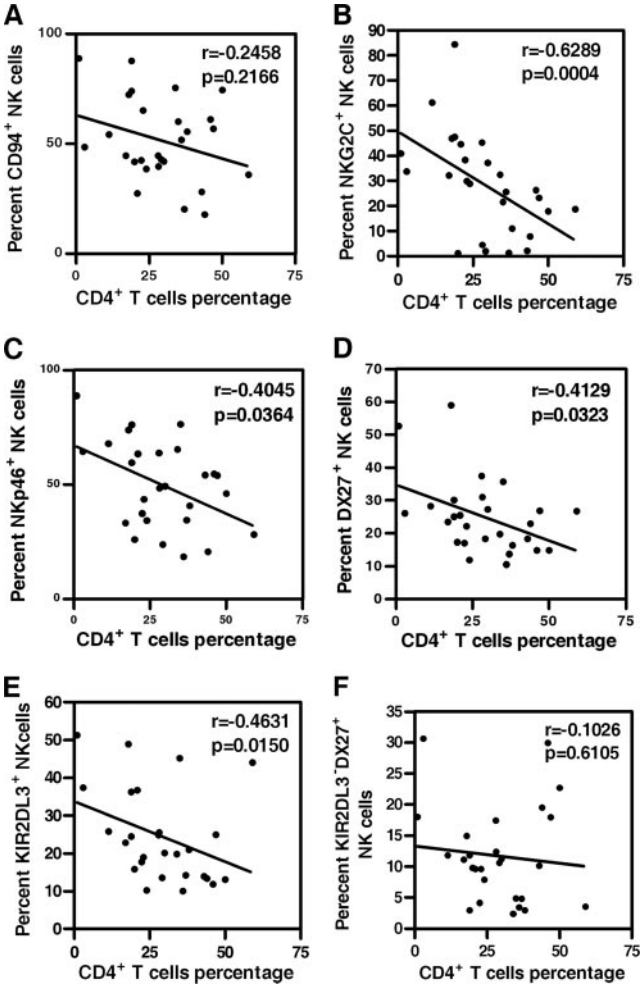

FIGURE 3. Correlation between the percentage of $CD4^+$ T cells and the frequency of NK cells expressing different NK receptors. *A*, Scatter diagram showing the relationship between the percentage of $CD4^+$ T cells and the frequency of NK cells expressing CD94. *B–D*, Diagrams showing a lower percentage of $CD4^+$ T cells in association with an increased percentage of NK cells expressing the NKG2C (activating receptor) (*B*), NKp46 (activating receptor) (*C*), and Dx27 Ab that recognizes KIR2DL2, KIR2DL3, and KIR2DS2. *E*, KIR2DL3 (inhibitory receptor) expression was also separately associated with lower percentage of $CD4^+$ T cells. *F*, There was no statistically significant correlation with the percentage of $CD4^+$ T cells when gating on $KIR2DL3^-DX27^+$ NK cells, showing that the association was solely due to KIR2DL3.

The expression of KIR2DL3, NKG2C, and NKp46 inversely correlates with the percentage of $CD4^+$ T cells in HIV-1-infected children

In this study we evaluated the correlation of the expression of activating and inhibitory receptors on NK cells with disease severity only in the HIV-1-infected patients as reflected by the percentage of $CD4^+$ T cells. The percentages of $CD4^+$ T cells, measured as part of the clinical follow-up of the HIV-1-infected patients, was available for 27 of the 28 samples tested from the HIV-1-infected patients, as was the viral load on each of the samples.

There was a statistically significant negative correlation between the percentage of $CD4^+$ T cells and the frequency of NK cells expressing the inhibitory KIR2DL3 receptor (Fig. 3*E*; $r = -0.4631$, $p = 0.0150$). In addition, the frequency of NK cells binding the DX27 Ab, which recognizes KIR2DL2, KIR2DL3, and KIR2DS2, also correlated negatively with the percentage of $CD4^+$ T cells (Fig. 3*D*; $r = -0.4129$, $p = 0.0323$). However, when $KIR2DL3^-DX27^+$ NK cells were plotted against the percentage of $CD4^+$ T cells, there was no statistical significance, determining that the correlation was solely due to the KIR2DL3 receptor and not to the other receptors recognized by the DX27 mAb (Fig. 3*F*). In addition, no such correlation was found with the frequency of NK cells expressing KIR3DL1 or KIR2DL1.

In this study, a lower percentage of $CD4^+$ T cells was correlated with a statistically significant higher percentage of NK cells expressing NKG2C (Fig. 3*B*; $r = -0.6289$, $p = 0.0004$). There was no correlation between the percentage of $CD4^+$ T cells and the percentage of NK cells expressing CD94 (Fig. 3*A*), NKG2A, or CD161.

We also evaluated the expression of both NKp44 and NKp46 and found a statistically significant increased frequency of NK cells expressing NKp46 with a lower percentage of $CD4^+$ T cells (Fig. 3*C*; $r = -0.4045$, $p = 0.0364$), but no such correlation between the low percentage of $CD4^+$ T cells and the percentage of NK cells expressing NKp44. However, when we analyzed the results of NKp44 and NKp46 by measuring their mean fluorescence intensity in correlation with the percentage of $CD4^+$ T cells, the statistically significant inverse correlation that existed between the percentage of $CD4^+$ T cells and $NKp46^+$ NK cells did not persist.

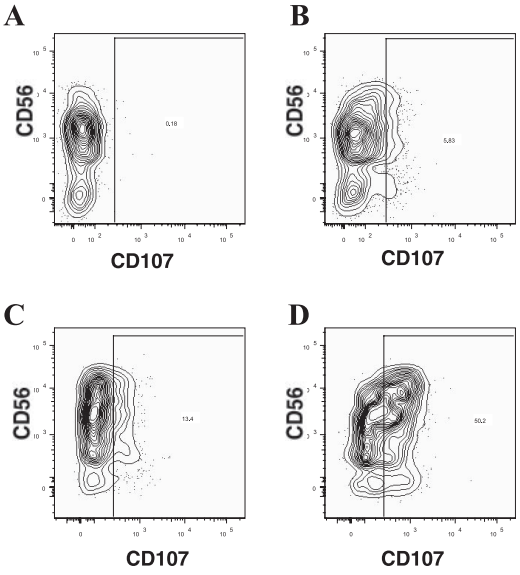

FIGURE 4. Dot plots comparing CD107 expression on NK cells. The gates were drawn based on fluorescence-minus-one. *A*, NK cells from an HIV-1-infected patient cultured with no stimulation. *B*, NK cells from the same patient in *A* cultured with K562 cells. *C*, NK cells from an EU subject cultured with no stimulation. *D*, NK cells from same subject in *C* cultured with K562 cells.

CD107 expression on NK cells

CD107 expression on NK cells was evaluated by culturing PBMCs with no stimulation and comparing them to PBMCs cultured with HIV-1 *gag/nef* pooled peptides, the cytokine IL-12, or the prototypic target for NK cells, the MHC-devoid K562 cell line. As expected and in agreement with other studies, the 28 HIV-1-infected children had a significantly higher expression of CD107 on their NK cells after stimulation with K562 ($p < 0.0001$) (Fig. 4*B*) or IL-12 ($p = 0.0064$) as compared with no stimulation (Fig. 4*A*) (46). CD107 expression after HIV-1 peptide stimulation was also higher than with no stimulation, but without reaching statistical significance ($p = 0.0503$).

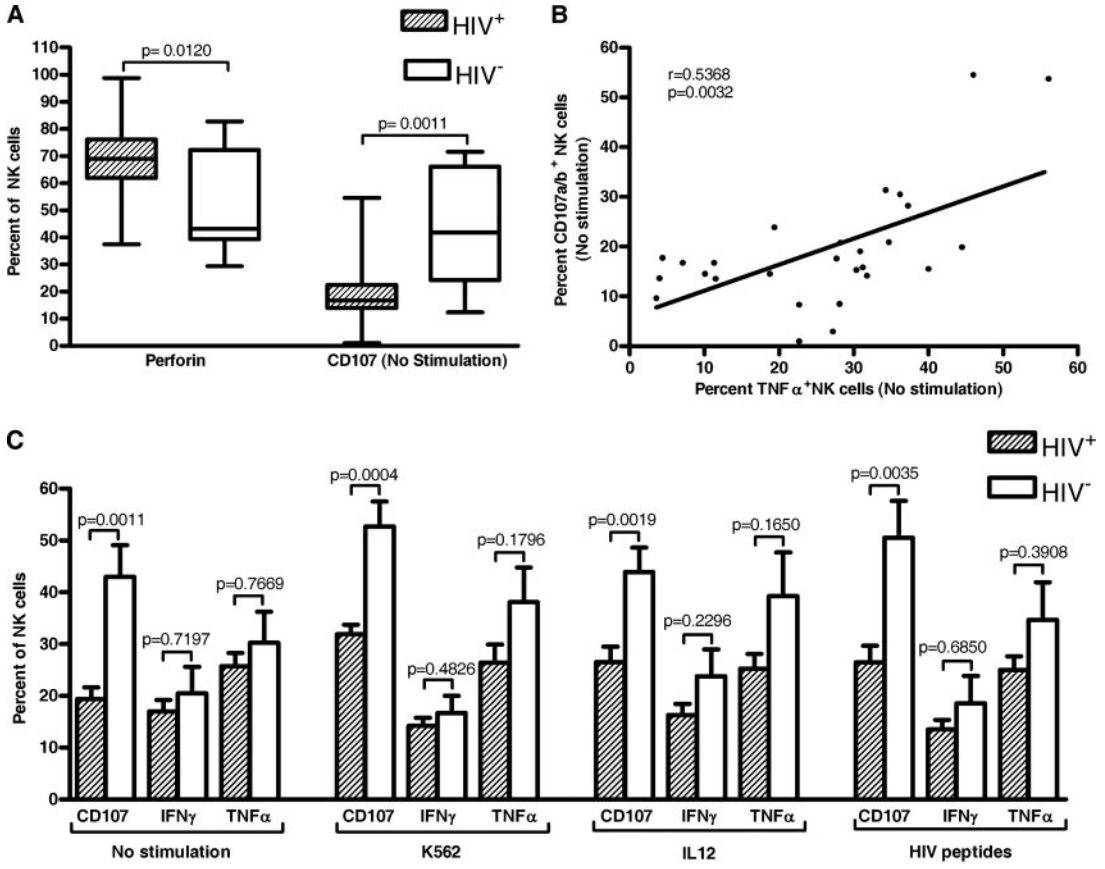

FIGURE 5. Functional comparison of NK cells from HIV-1-infected and HIV-1-EU children. *A*, Lower expression of CD107 on NK cells from HIV-1-infected (HIV^+) children compared with HIV-1-EU (HIV^-) children after no stimulation whereas a higher frequency of NK cells express perforin in HIV^+ children compared with HIV^- children. *B*, CD107 correlates with TNF-α expression in NK cells from HIV-1-infected children with no stimulation. *C*, CD107, TNF-α, and IFN-γ expression with different stimulations.

In addition, when comparing CD107 expression on NK cells between HIV-1-infected children and the HIV-1-EU children, there was a consistently lower level of expression in the HIV-1-infected group regardless of the stimulation used (Figs. 4 and 5*C*). However, intracellular staining for perforin, without stimulation, showed a higher percentage of $perforin^+$ NK cells in the HIV-1-infected patients compared with the EU children (Fig. 5*A*).

We also assessed NK function by intracellular cytokine staining for IFN-γ and TNF-α. We did not find any significant difference in the expression of either cytokine between the different stimulation groups or between the HIV-1-infected children and the EU subjects (Fig. 5*C*). However, there was a correlation between the level of expression of CD107 and that of TNF-α in the HIV-1-infected children with no stimulation or with HIV-1 peptide stimulation (Fig. 5*B*).

Discussion

The innate immune system, including NK cells, is thought to be a major contributor to viral elimination in some acute viral infections before the development of the adaptive immune response (57–59). There is accumulating evidence of a role for NK cells in the control of chronic HIV-1 infection in adults (38, 46, 60–63). However, our knowledge surrounding NK cell function in pediatric populations in general and in perinatally HIV-1-infected children in particular is limited (51). In this cross-sectional study we evaluated NK cell frequency, number, phenotype, and function and their relation to the clinical status in HIV-1-infected children relative to EU children. We found no difference in the numbers of NK cells or NK cell subpopulations between these groups. Moreover, there was no difference in those numbers even when the infected patients were divided into viremic and aviremic groups. However, when compared with the HIV-1-infected children, the frequency of KIR3DL1- and KIR2DL1-expressing NK cells was reduced in the EU children. We also found a strong inverse correlation between the percentage of $CD4^+$ T cells, which has been documented as a better predictor of clinical progression than plasma viral load in HIV-1-infected children, and the frequency of NK cells expressing the activating receptors NKG2C and NKp46 and the inhibitory receptor KIR2DL3 (64). In addition, the HIV-1-infected children had a lower number and percentage of CD107-expressing NK cells compared with the EU children. This functional difference, reflected by CD107 expression, was seen not only at baseline but also after stimulation with HIV peptides, IL-12, and K-562 cells. Conversely, the HIV-1-infected children had a higher number and percentage of $perforin^+$ NK cells compared with the EU children.

The reports on the number of NK cells in HIV-1-infected adult individuals are inconsistent, with some studies reporting a depletion in the number of NK cells in infected patients as compared with healthy uninfected controls (51). In this study we compared the absolute numbers and the percentages of NK cells within the lymphocyte population from PBMCs in perinatally HIV-1-infected children to those in HIV-1-EU children. Surprisingly, we did not detect any difference between the total number or the subsets of NK cells between the infected group and the EU group, even when evaluating for the effect of viremia (46). The lack of NK cell depletion in perinatally infected children vs infected adults may

reflect the enhanced regenerative capacity of the pediatric innate immune system, the reduced cumulative exposure to viruses and other pathogens that might exhaust the NK cell system, or NK cells that are more refractory to apoptosis or other forms of cell death and turnover. In addition, other than the possible difference in production or destruction of NK cells between children and adults, a third mechanism might be a different distribution and sequestration of the NK pool in the lymphoid tissue of children when compared with that of adults.

Recent reports have shown an increased proportion of NK cells that express the CD94/NKG2C heterodimer, an activating complex, in HIV-1-infected adult patients (65). Further studies have examined the expression of NKG2A vs NKG2C in relation to HIV-1 and HCMV infection and demonstrated that the higher proportion of $NKG2C^+$ NK cells in HIV-1-infected patients was attributable to the higher proportion of HCMV infection in this population of subjects as compared with HIV-1-uninfected individuals (65, 66). In this study there was a trend toward an increase in the frequency of NK cells that express both types of NKG2 receptors, the inhibitory NKG2A and the activating NKG2C, in the HIV-1-infected children. Interestingly, the HCMV status in this study group did not affect the expression of NKG2C or NKG2A on the NK cells. HCMV seropositivity data was not available for all the HIV-1-EU children, and therefore we could not compare the total effect of HCMV on NK cells from the infected children to NK cells from the EU ones. Azzoni et al. have reported a persistent depletion of $CD161^+CD56^+CD16^+$ NK cells after viral suppression and immune reconstitution in HIV-1-infected children (51). In this study, in contrast, we found no difference in the expression of CD161 between the viremic and the aviremic children or between the HIV-1-infected and EU children.

A recent report showed that seronegative, highly exposed, uninfected female sex workers possessed activating KIR genotypes whereas the HIV-1-infected female sex workers possessed the corresponding inhibitory KIR genotypes (37). Interestingly, in this study we showed that a lower number and percentage of NK cells from the EU children expressed inhibitory KIR (KIR3DL1, KIR2DL1, and KIR2DL3) as compared with the HIV-1-infected children. This is in agreement with the Jennes et al. study, implying a potentially protective role of a subset of inhibitory KIR^- NK cells and HIV-1 nontransmission (37).

Impaired NK cell cytotoxic function in adult viremic HIV-1 infection has been partly associated with the reduced surface expression of NCRs (NKp30, NKp44, and NKp46) (32). Our results showed no correlation between viral load and NCR expression, although there was an increase in $NKp46^+$ NK cells in association with declining percentages of $CD4^+$ T cells.

To evaluate the effect of HIV-1 disease stage on NK cells, we assessed the relationship between the percentage of $CD4^+$ T cells and the change in NK cell subsets. We found an inverse correlation between the percentage of $CD4^+$ T cells and the frequency of NK cells expressing both the activating receptors NKG2C and NKp46 and the inhibitory receptor KIR2DL3. However, the NKp46 mean fluorescence intensity did not correlate with the $CD4^+$ T cell depletion. One possible explanation for this finding would be that the drop in the percentage of $CD4^+$ T cells correlates with an expansion of $NKp46^+$ NK cells without an increase of the expression of NKp46 receptors on the surface of individual NK cells. The strongest correlation of $CD4^+$ T cell depletion and NKG2C expression on NK cells ($p = 0.0004$) might suggest a shift in the equilibrium between inhibitory and activating receptors toward a net activating signal with a dropping percentage of $CD4^+$ T cells. Vieillard et al. have shown that the expression of the ligand for NKp44 on $CD4^+$ T cells is induced by a highly conserved HIV-1 gp41 peptide, which makes them more susceptible to NK lysis (16). In addition, Bonaparte and Barker have shown that NK cells from healthy individuals that do not express inhibitory KIR and NKG2A can kill HIV-1-infected autologous $CD4^+$ T cells. However, the ability of NK cells to lyse HIV-1-infected autologous $CD4^+$ T cells is minimal (67, 68). This association between the expression of NKG2C and NKp44 on NK cells and the low $CD4^+$ T cell percentage cannot be explained without evaluating the ex vivo effect of NK cells on autologous HIV-1-infected $CD4^+$ T cells, which is not in the scope of this study. The net effect of this change in surface marker expression on the function of NK cells was not clear, as there was no evident correlation between CD107, IFN-γ, TNF-α, or perforin expression and the percentage of $CD4^+$ T cells.

Other studies have shown that NK cells in HIV-1-infected patients expressed low levels of perforin in untreated groups but levels comparable to those of healthy controls in the HIV-1-infected, treated aviremic group (69, 70). In our study, a significantly higher frequency of NK cells in highly active antiretroviral therapy (HAART)-treated HIV-1-infected children expressed perforin compared with those in EU children. In addition, the expression of CD107a/b (LAMP-1/2) on the surface of $CD8^+$ T cells and NK cells is a sign of the recent degranulation of intracellular vesicles by these lymphocytes and an indirect method for evaluating the potential cytotoxic activity of these cells (46, 52, 53, 71). We found a lower CD107 expression in the HIV-1-infected children relative to the EU children at baseline and with K562, IL-12, and HIV peptide stimulations. This discordance in the expression of CD107 and perforin could be explained by the fact that the NK cells that do not degranulate and, hence, do not express CD107 will have more accumulation of perforin in the intracellular granules (71). Of note is that even though the study subjects represent a very heterogeneous group in terms of their antiretroviral regimens, they were all chronically infected (infected since birth) and treatment-experienced patients. Taken together, these results imply a decreased activity of NK cells in HIV-1-infected children.

To our knowledge, these results represent the first detailed comparative analysis of NK cells in perinatally HIV-1-infected children compared with an EU control group. The lower level of degranulation along with the increased level of expression of inhibitory receptors on NK cells in HIV-1-infected children imply that chronic HIV-1 infection leads to an accumulation of dysfunctional NK cells, even when the number and frequency of certain NK cell subsets are preserved. This represents a difference from HIV-1-infected adults in whom, even though the total NK cell numbers and frequencies are preserved, there are significant changes in the distribution of their NK cell subsets compared with HIV-1-uninfected control subjects (72, 73). This relationship did not appear to be affected by the degree of viremia or indirectly affected by the type of treatment regimen or level of adherence of each subject. The role of these findings in mother to child transmission of HIV-1 would be best evaluated by a comparison of the EU group with healthy unexposed children and a control for maternal treatment that were not part of this study. Although the $CD4^+$ T cell percentage decline is associated with a repertoire of cell surface inhibitory and activating receptors favoring NK cell activation overall, there was no observed correlation between the $CD4^+$ T cell percentage and the level of degranulation of NK cells as measured by the expression of CD107; this represents a disconnect between the expression of activating and inhibitory receptors on NK cells and their possible function as measured by their level of degranulation. Lastly, the patients being compared in this study were similar in terms of age, gender, and race distribution, and the observed differences in their NK cells could only be explained by

the effect of HIV-1 infection. In summary, these results show decreased activity, but not frequency, of NK cells in perinatally HIV-1-infected children.

Acknowledgments

We thank all of the HIV-1-infected and exposed uninfected participants and their parents or legal guardians and their doctors at the Jacobi Medical Center for their participation in this study. We also thank Keith Garrison, Lishomwa Ndhlovu, Jennifer Snyder-Cappione, and Christian Willberg for their critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

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