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Sanjana Mahapatra, Ph.D., William T. Shearer, M.D., Ph.D., Charles G. Minard, Ph.D., Emily Mace, Ph.D., Mary Paul, M.D., Jordan S. Orange, M.D., Ph.D



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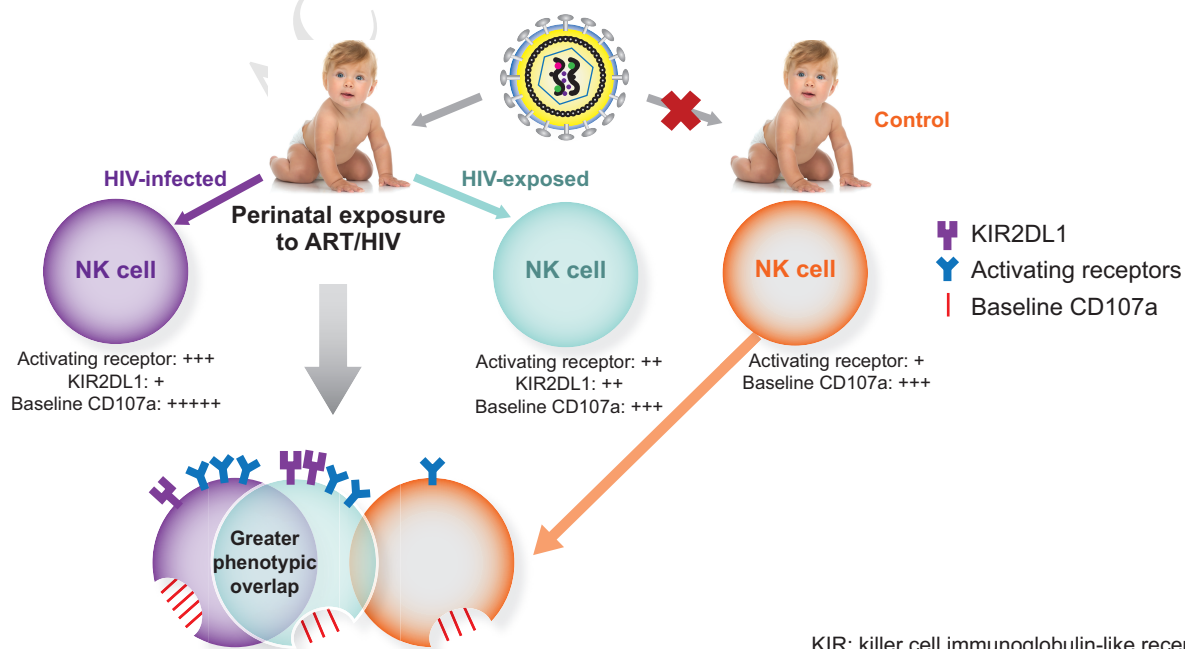
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NK cells from treated HIV-infected children display an activated phenotype



NK cells in treated HIV-infected children display altered phenotype and function

Short title: NK cell phenotype in HIV-infected children

Sanjana MAHAPATRA^{a,b}, Ph.D., William T. SHEARER^{b,d}, M.D., Ph.D., Charles G. MINARD^c, Ph.D., Emily MACE^{a,b,1}, Ph.D., Mary PAUL^d, M.D., and Jordan S. ORANGE^{a,b,d,1}, M.D., Ph.D.

^aDepartment of Pathology and Immunology, Baylor College of Medicine, Houston, Texas, United States of America;

^bCenter for Human Immunobiology, Texas Children's Hospital, Houston, Texas, United States of America;

^cDepartment of Medicine, Baylor College of Medicine, Houston, Texas, United States of America;

^dDepartment of Pediatrics, Baylor College of Medicine, Houston, Texas, United States of America;

¹Present address: Department of Pediatrics, Vagelos College of Physicians and Surgeon, Columbia University, New York, New York, United States of America

Corresponding author: Jordan S. Orange, CUMC/NewYork-Presbyterian Morgan Stanley Children's Hospital; 3959 Broadway, New York, NY 10032; E-mail: jso2121@cumc.columbia.edu; Tel: 212-305-2934; Fax: 212-305-3020

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Abstract

Background: Chronic human immunodeficiency virus (HIV) infection is known to trigger a population redistribution and alteration in the functional capacity of NK cells. Due to improved anti-retroviral treatments, there are rising numbers of adolescents and young adults worldwide that are living with HIV infection since birth.

Objective: We sought to determine how NK cell phenotypic and functional subsets are altered in treated pediatric patients.

Methods: NK cells were contrasted among 29 HIV-unexposed and uninfected controls (5-19 years), 23 HIV-exposed but uninfected patients (HEU) (3-19 years) and 25 HIV-infected patients (3-19 years) using multi-parametric flow cytometry.

Results: While majority of the NK cell markers did not differ, activating receptors like NKp46, DNAM-1, NKG2C and stimulatory receptors like CD2 and CD11c were expressed by a higher frequency of NK cells in HIV-infected patients than controls. Interestingly, there were less differences between HIV-infected and HEU children. There was an inverse relationship between CD4/CD8 T cell ratio (as a marker of disease progression) and CD11c and NKG2C frequency and CD69 upregulation on stimulation among HIV-infected patients.

Conclusions: A chronic NK cell activation phenotype persists in HIV-infected children receiving anti-retroviral therapy and is associated with declining CD4/CD8 T cell ratios. A lower CD4/CD8 T cell ratio was associated with higher baseline granzyme B ($p=0.0068$, $R^2=0.29$) and degranulation potential ($p=0.022$, $R^2=0.22$) in stimulated NK cells. Thus, NK cells in HIV-infected children receiving treatment have reduced functional potential and an activated phenotype that distinguishes them from uninfected children.

Clinical implications:

- NK cells persist in an activated state in perinatally HIV-infected children regardless of age
- Similarities in NK cell phenotype between HIV-exposed and HIV-infected children suggest an impact upon NK cells through HIV-exposure

Capsule summary: This is the first high-resolution NK cell phenotyping study in HIV-infected children that demonstrates an activation phenotype in NK cells despite anti-retroviral therapy. It also establishes an impact upon NK cells in both HIV-infected and exposed but uninfected children.

Keywords: NK cell, treated HIV, flow cytometry, children, vertical exposure

Abbreviations: HIV (human immunodeficiency virus), HEU (HIV-exposed but uninfected), NK (natural killer), MHC-I (major histocompatibility complex-I), DNAM-1 (DNAX accessory molecule-1), IFN (interferon), ADCC (antibody dependent cellular cytotoxicity), ART (anti-retroviral therapy), MTCT(mother-to-child transmission), CCL (chemokine (C-C motif) ligand), CCR (chemokine (C-C motif) receptor), MFI (median fluorescent intensity), KIR (killer cell immunoglobulin-like receptor), HLA (human leukocyte antigen), KLRG1 (killer cell lectin-like receptor subfamily G member 1), hCMV (human cytomegalovirus)

Introduction

Natural killer (NK) cells are lymphocytes of the innate immune system that kill virally-infected and malignant cells. They arise in the bone marrow, undergo central and peripheral development and ultimately attain functional competency, with the mature subsets in the majority found in the peripheral blood. NK cells are armed with an array of activating, inhibitory and adhesion surface receptors, which determine the population's phenotypic diversity and function^[1, 2]. During viral infections, NK cells can recognize virally infected cells through their activating receptors, or the sensing of decreased MHC-I molecules through their cognate inhibitory receptors^[3]. They can also be activated by stress-induced ligands that bind to stress-sensing receptors such as NKG2D. Once activated, NK cells mediate antiviral immunity via direct killing of infected cells, production of soluble antiviral mediators such as IFN γ , and promoting/facilitating broader immunity. Direct killing includes CD16-dependent antibody-dependent cellular cytotoxicity (ADCC) and natural cytotoxicity both via the secretion of preformed lytic granules containing perforin and granzyme. Accordingly, NK cells can contribute to the control of viral infections and evidence exists for their role in the defense against HIV^[1, 2].

Chronic HIV infection is characterized by phenotypic redistribution of NK cell subsets that potentially compromise antiviral functions. Defective NK cell killing has been identified in the context of disease progression and defective ADCC. Diminished secretion of soluble mediators has also been reported in HIV-infected patients^[4]. This could be owing to expansion of a dysfunctional CD56⁻ NK cell subset that has impaired cytotoxicity and cytokine release^[5, 6]. Abnormal cytotoxicity has additionally associated with downregulation of activating receptors in viremic patients^[7], but increased NK cell activity has been identified in some viremic patients^[8]. Irrespective, NK cell phenotypic changes have been linked to ongoing viral replication that is

partially recovered following viral suppression through anti-retroviral therapy (ART)^[9]. However, IFN γ secretion from NK cells is reduced in aviremic patients and activated NK cells were observed in HIV-infected patients regardless of ART^[10, 11]. Thus, ART does not fully reconstitute NK cell phenotype and function, and, exact characteristics of residual NK cell subset abnormalities in treated patients warrant further investigation.

While most studies of NK cells in HIV-infected patients have been conducted in adults, few studies in HIV-infected children have identified some limited NK cell phenotypic changes that persist despite treatment^[12, 13]. Since we have previously shown that a high-resolution NK cell phenotype in children differs from adults^[14], we pursued this level of detail in treated HIV-infected children. We were especially focused upon mother-to-child transmission (MTCT) cases as most children are on ART since birth^[15]. Moreover, perinatal transmission does not occur in 65-85% of infants, even in the absence of ART, leaving children HIV-exposed but uninfected (HEU). HEU infants in the context of MTCT can potentially provide important insights into NK cell antiviral defense as the HIV-exposed NK cells produce increased CCL3-5 which can suppress viral replication through blocking the cognate CCR5^[16]. Furthermore, activated and perforin-expressing NK cells decline over the first year of life in HEU infants but not in HIV-unexposed controls, indicating some impact upon and functional differences in NK cells from HEU children^[17]. Therefore, we set out to determine how NK cell subsets at relatively high resolution might be altered and what distinguishing characteristics, if any, they have in HEU children. We identified differences in NK cell phenotype and function in both cohorts and could associate the phenotypic variation with markers of disease progression in HIV-infected patients. Given the prevalence of pulmonary complications in HIV-infected patients^[18] and the role of NK cells in allergic asthma^[19], we also examined NK cell phenotype based on asthma incidence.

Methods

Human subjects and sample preparation

A total of 77 subjects (aged 3 to 19 years) were recruited at Texas Children's Hospital (Table I). All individuals provided written informed consent approved by the Institutional Review Board of Baylor College of Medicine for the protection of human rights. Peripheral blood mononuclear cells (PBMCs) were extracted from whole blood using Ficoll-Paque (ThermoFisher Scientific, Waltham, MA) density gradient centrifugation and cryopreserved in filter-sterilized heat-inactivated fetal bovine serum (HI-FBS) (Atlanta Biologicals, Flowery Branch, GA) containing 10% dimethyl sulfoxide (ThermoFisher Scientific, Waltham, MA).

HIV-infected patients were recruited from Retrovirology clinic and controls from the Allergy and Immunology clinic^[14]. HEU subjects were recruited from those enrolled in the Pediatric HIV/AIDS Cohort Study^[18]. Inclusion criteria was mother-to-child transmission for HIV-infected and perinatal exposure for HEU children.

Antibodies

All commercial antibodies were obtained from sources as previously outlined^[14].

Cell staining, acquisition and data analysis

Cryopreserved PBMCs were thawed and stained as previously described^[14]. Briefly, cells were suspended at a concentration of $\sim 4 \times 10^6$ cells/ml. For surface staining (Panels 2-4) (Table S1), cells were stained in PBS (ThermoFisher Scientific, Waltham, MA) 2% FBS buffer

with a cocktail of antibodies for 15-20 min in the dark. For Panels 1 and 5, cells were stimulated with phorbol 12-myristate 13-acetate (10 ng/ml, Sigma-Aldrich, St. Louis, MO) and ionomycin (1 µg/ml, Sigma-Aldrich, St. Louis, MO) and incubated for 4 hours. Cells were washed and fixed with 1X PBS containing 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) at the end of staining.

A modified LSR Fortessa (BD Biosciences) was used to acquire ~2000 NK cells for each donor. Data analysis was conducted using Kaluza (Version 1.3, Beckman Coulter) with the gating strategy illustrated earlier^[14].

Statistics

Comparisons across 3 groups were made using the Kruskal-Wallis test. If the overall test for differences was significant at the 0.05 level, then all pairwise comparisons were assessed using Wilcoxon rank-sum test with Bonferroni adjustment for multiple comparisons (STATA Version 14, Stata Corp). Two-group comparisons were assessed using the Wilcoxon rank sum test. Staining, acquisition and analysis was repeated for 3 controls and inter-experimental variability for controls was determined using Bland-Altman analysis (Prism, Version 6, GraphPad Software, Inc.). Variability was defined as the mean difference in percentages for each marker in Panels 2-4. Combinatorial subsets were identified at a minimum threshold of 3%. The mean values were compared between HIV-infected and HEU cohorts using a general linear mixed model (SAS, Version 9.4, SAS institute)^[14].

Results

NK cells in treated HIV-infected pediatric patients display an activated phenotype.

Chronic HIV infection triggers an abnormal redistribution of NK cell subsets, which does not fully recover post ART. The mature CD56⁺CD16⁺ NK cell subset is selectively depleted in HIV-infected adolescents with low CD4⁺ T cell counts^[20] and HIV-infected patients have lower levels of NK cell degranulation despite >3 years ART^[13]. Therefore, we sought to evaluate if functional and phenotypic NK cell defects persist in HIV-infected children on long-term therapy using high-resolution multiparametric flow cytometry. Our cohort included 25 MTCT perinatally HIV-infected children (3-20 years), 23 perinatally HEU children (3-20 years) and 29 demographic-matched control subjects (5-20 years). Since NK cell development and effector functions are dependent on a range of activating, adhesion and inhibitory receptors, progressively acquired or lost through different stages of maturation, we utilized 5 previously designed panels that encompass the spectrum of NK cell biology and function (Table S1)^[14].

These panels were used to distinguish the frequency of NK cell markers among our three cohorts. Panels 2-4 addressed baseline expression of surface markers whereas panels 1, 5 characterize both baseline characteristics and changes following stimulation with PMA and ionomycin. Although the overall NK cell frequencies did not differ between our study groups (data not shown), certain activating, adhesion and stimulatory receptors were elevated in HIV-infected children (Fig 1). Baseline frequency of DNAM-1, CD2 and NKp46 was elevated in both HIV-infected and HEU subjects compared to controls (Fig 1A, F, G). In contrast, NKG2C was specifically increased in HIV-infected individuals (Fig 1C). While this has been repeatedly

demonstrated in HIV-infected adults^[21, 22], it is less established in perinatally-infected children. NK cell frequency of CD11c and ability to upregulate activation marker CD69 post-stimulation was also higher in HIV-infected children (Fig 1E, J). However, baseline frequency of CD16, CD69 (Fig 1D, I) and CD11b, 2B4, CD18 (data not shown) and stimulated expression of NKp46 (Fig 1H) were reduced in HIV-infected patients while NKp30 was unaffected (Fig 1B). While the HIV-infected and HEU cohorts were processed and acquired in a paired manner, the demographic-matched control subjects were from our prior study and used here for comparison^[14]. To evaluate any impact this might have had, Bland-Altman analysis was performed to estimate agreement between the older set of experiments for 3 random controls (out of 29 total patients) and the experiments with the same parameters used for HIV-infected and HEU children. The maximum difference in frequency for any one fluorochrome between the 2 sets of experiments was <10% (Table S2A). The difference for each fluorochrome was accordingly applied to the frequency values of all 29 controls for each marker such that the values would be skewed towards those of HIV-infected patients (Table S2B). Of all the activating receptors increased in HIV-infected patients, only DNAM-1 was no longer significant on repeating the statistical analysis. Given the validity identified through these analyses, we have utilized the historical age-matched control cohort^[14] for comparison to the HIV-infected and HEU cohorts for all frequency comparisons. This agreement study was also repeated for median fluorescent intensity (MFI). Here the variation identified was >10%, and thus MFI was only contrasted in further analyses between HIV-infected and HEU children and revealed no significant differences for any marker (data not shown).

Since there was identified disparity in frequencies of the individual receptors among our cohorts, we additionally contrasted the simultaneous expression of these markers between HIV-infected and HEU children. Treeplot analysis was used to generate the frequencies of all 256 combinations of 8 markers in each panel. Although most combinatorial subsets did not differ,

the KIR2DL1⁺ subset that lacked all other inhibitory receptors like CD158b, CD158e, NKG2A, KLRG1 and activating receptors like KIR2DS4 and NKG2C, was significantly lower in HIV-infected patients (Fig 2). Interestingly, NK cells of the only patient with a viral load >3 logs (Table S3) in our cohort, had the lowest KIR2DL1⁺ subset frequency (0.05%). Few studies have indicated that HIV-infected patients with KIR2DL1⁺ NK cells who also express the cognate HLA-C2 ligand have enhanced NK cell activity, while the presence of KIR/HLA discordance provides a protective advantage against HIV^[23-25]. Of the 10 HIV-infected patients we performed HLA typing on, 9 were mismatched to KIR2DL1 (data not shown). Therefore, one explanation could be that this mismatch did not protect against HIV due to low KIR2DL1 expression. That said, our cohort is underpowered for such considerations as these hypotheses are only resolvable in larger population studies. Furthermore, conclusions cannot be drawn since we do not have the HLA haplotype of all patients. Thus, HIV-infected patients not only have elevated expression of individual activating receptors but also decreased expression of a combinatorial inhibitory subset. Although the wide age range of our patients may confound these results, we do not believe this to be a major limitation as we have included age-matched controls. Moreover, our previous study noted far fewer differences among NK cells of healthy children than between children and adults^[14].

Reciprocal relationship between NK cell activating receptors and CD4/CD8 T cell ratio.

HIV infection is characterized by chronic immune activation that endures despite suppression of viral replication with persistent evidence of T cell activation^[26]. CD4/CD8 T cell ratio is used as a reliable biomarker of disease progression in HIV infection since it is more stable than CD4⁺ T cell counts and normalizes gradually during ART^[27, 28]. CD4/CD8 ratio is

also posited to be an indicator of T cell activation, senescence and inflammation. Since our data alludes to an NK cell activation phenotype in HIV-infected patients, we hypothesized that declining CD4/CD8 ratio associates with frequency of activating receptors on NK cells. Indeed, CD69 upregulation by NK cells post-stimulation negatively associated with CD4/CD8 ratio (Fig 3A). The same correlation was observed on a per cell basis as a change in CD69 MFI negatively associated with both CD4/CD8 ratio and frequency of CD4⁺ T cells (Fig S1A-B). Activating and stimulatory receptors like KIR2DS4, CD244, NKG2C and CD11c also had a reciprocal relationship with CD4/CD8 ratio (Fig 3B-E). Only NKG2C was significant when the same markers were tested against CD4⁺ T cell counts, underscoring the value of using CD4/CD8 ratios (Fig S2D). CD69, NKG2C and CD11c were particularly relevant as they were significantly higher in HIV-infected patients than controls (Fig 1). Moreover, CD94, which associates with NKG2C also had a reverse association with both CD4⁺ T cell frequency and count (Fig S1C-D). Thus, NK cell activating receptors are more highly expressed in HIV-infected individuals having greater disease severity.

CD57 is a terminal maturation marker on NK cells that can be acquired after cytokine stimulation *in vitro*^[29]. Its expression has been reported to increase with aging as well as in chronic infections like hCMV and HIV^[30]. In our cohort, the CD4/CD8 ratio was linked to the variation in CD57 ($R^2=0.5$) and had the strongest inverse relationship of all markers tested (Fig 3F). This pattern was repeated with both frequency and CD4⁺ T cell count as well as between CD57 MFI and CD4/CD8 ratio (Fig S3). Although CD57 frequency did not differ amongst the three cohorts we studied (data not shown), high CD57 expression on NK cells was associated with disease severity. This increased NK cell maturation could be due to the pro-inflammatory cytokine environment in active HIV disease or hCMV coinfection, both of which can also mediate chronic immune activation in HIV-infected individuals^[26].

In some studies, impaired NK cell activity and degranulation potential during HIV infection is not recovered post ART^[13, 20]. In our cohorts, NK cells in HIV-infected children had significantly higher baseline IL-5 and CD107a expression (Fig 4A, C). However, the increased CD107a expression was not retained after stimulation (Fig 4D). IL-5 presumably did not increase on stimulation (Fig 4B) due to lack of IL-2 in addition to PMA/ionomycin stimulation as previously shown^[31]. On repeating analysis, the increased baseline CD107a expression in HIV-infected individuals was still statistically significant from control donors but not IL-5 (Table S2B). Interestingly, the stimulated but not baseline levels of CD107a and granzyme B were inversely associated with CD4/CD8 ratio and CD4⁺ T cell counts (Fig 4E, F, S4A). Similar trends existed between increase in CD107a MFI after stimulation and CD4/CD8 ratio as well as granzyme and CD4⁺ T cell frequency but not count (Fig S4B, S5). Thus, the HIV-infected children with the lowest CD4/CD8 ratio had the highest baseline granzyme levels and exhibited greatest degranulation potential on stimulation, implying that chronic activation owing to disease severity was promoting NK cell activation state.

We also evaluated viremia as an association for the activated NK cell phenotype observed in HIV-infected patients. Of the 25 patients in our cohort, 12 had detectable viral loads but only one had viral load >3 logs (Table S3). One patient had <200 CD4⁺ T cells and clinically defined to have AIDS but was not excluded from the analysis. On comparing the NK cell phenotype, the frequency of CD18 and granzyme B at baseline and MFI of CD11a were significantly higher in patients with detectable viral loads (Fig S6). Since our cohort had diverse ART regimens, we also evaluated phenotypic differences by treatment. NK cells in patients that received a combination of integrase and nucleoside reverse transcriptase inhibitors had the highest CD11b MFI (data not shown). Viremia and medication regimen therefore might provide some influence upon the activated NK cell phenotype found in HIV-infected children.

We also considered asthma as an inflammatory disease in our patients that could potentially affect NK cell phenotype as an increased asthma burden has been documented in HIV-infected patients since onset of ART era^[32]. NK cells in the 6 HIV-infected patients with asthma had higher frequency of IL-15 receptor- α chain, KIR2DS4 and CD54 but lower CD158e (Fig 5). Thus, the incidence of asthma may contribute to but does not account for the activation phenotype in HIV-infected patients.

NK cell subsets of HIV-exposed and HIV-infected patients are phenotypically similar

It is increasingly appreciated that HEU individuals have specific characteristics. For example, an increased incidence of respiratory infections in HEU children has been reported in South Africa and India^[33]. Immunologically, HEU children have a more mature and activated phenotype compared to HIV-unexposed children. These studies suggest that exposure to HIV and/or ART drives phenotypic changes that persist beyond infancy. To examine this, we compared the NK cell phenotype in HEU children to that in both HIV-infected patients and controls. While several activating receptors were upregulated in HIV-infected patients, some such as NKp46 and DNAM-1 were also increased in HEU children (Fig 1A, G). Moreover, NKp30 and inhibitory receptor KLRG1 only differed between HEU subjects and controls suggesting a potential correlate for HIV infection resistance within the NK cell population of children (Figs 1B, S7A). Thus, HEU children have elevated activating receptors but fewer than those in HIV-infected patients.

Interestingly, CD122 was highest in HEU children (Fig S7B). NK cells of HEU adults have been previously reported to have higher CD57 than HIV-infected patients^[34]. Although the

results were not significant, the frequency of CD57 alone (data not shown) and simultaneous expression of CD57, CD122, CD11b and CD16 trended towards being higher in HEU children (Fig S7C).

On tabulating the top combinatorial subsets by panel, only the developmental subset differed between HIV-infected and HEU children due to lower CD57 while the remaining subsets were overlapping between the two cohorts (Table S4). Thus, higher expression of CD122 and developmentally mature NK cell subset convey a more mature phenotype in HEU children compared to HIV-infected patients.

All NK cell markers that differed among our 3 cohorts are summarized in Fig 6. The baseline frequency of NKp46 was selectively reduced in controls in contrast with elevated NKG2C and baseline degranulation in HIV-infected children. Several markers only significantly differed between two cohorts. For example, NKp30 was significantly different between HEU children and controls. However, the HIV-infected cohort was not significantly different from either the HEU or control cohorts. Altogether, HIV-infected and HEU children had the most similarities in NK cell marker frequency followed by HIV-infected and controls. Given the degree of overlap in top NK cell subsets between HIV-infected and HEU children (Table S4), overall, they appear to be more phenotypically similar than HEU children and controls.

Discussion

While MTCT rates are 1-2% in developed countries, pediatric HIV infection remains a significant problem in low-income countries^[15]. Given the likely importance of NK cells in HIV pathogenesis, maintaining NK cell integrity contributes to controlling disease progression. However, reports about restoration of NK cell subsets post ART have been inconsistent and studies were limited in the NK cell markers examined^[9, 13, 35]. Therefore, we comprehensively characterized NK cell phenotype in both HEU and HIV-infected children to better understand NK cell alterations. We identified a predominant activation phenotype in the NK cells of HIV-infected pediatric patients as well as several activating receptors that were inversely associated with CD4/CD8 T cell ratio.

Chronic immune activation in HIV infection is linked to shorter survival^[26]. Studies in treated adults have shown that persistent NK cell activation is indicated by high CD38 and low FcRγ^[11, 36]. To our knowledge, the work presented here is the first to demonstrate elevated NK cell activating and stimulatory receptors in pediatric patients. Potential mechanisms behind immune activation include reactivation of infections such as CMV and hepatitis C as CMV intervention has been shown to reduce T cell activation in HIV-infected patients^[26, 37]. Inflammation marked by IFN-I production could also activate NK cells^[38]. Similarly, destruction of mucosal membrane could release microbial products that activates innate immunity promoting inflammation, further driving systemic activation^[26]. Finally, while low-level viremia has been associated with chronic T cell activation, a similar link has not been established with regards to NK cells but could explain some of the phenotype in our patient cohort (the majority of which are aviremic).

Most of the activating receptors were elevated in relation to healthy controls in our studies and not HEU. Thus, our data revealed greater similarities between HIV-infected and HEU subjects than with controls (Fig 6, Table S4). HEU infants mount immune responses that potentially protect them from HIV infection^[33]. Accordingly, higher KIR2DL1 subset and CD122 in HEU individuals may suggest correlates of protection (Figs 2, S7). However, a more activated and memory phenotype associated with increased mortality and morbidity has also been reported in HEU infants compared to controls^[33]. This might explain the phenotypic similarities between HEU and HIV-infected children. Exposure to HIV or viral particles *in utero* potentially created an immune imprint that persisted beyond infancy which could in turn account for the increased disease burden in HEU adolescents^[33]. The contributions of ART and HIV exposure, however, are difficult to disentangle. Although we used Bland-Altman analysis to evaluate the usability of significant results from previous data, it may be inadequate for estimating the values of all controls, which needs to be explicitly stated as a limitation of our study.

Understanding NK cell phenotype and function in the context of HIV is likely to be key to optimizing strategies for disease treatment. While it is unclear whether an NK cell activation phenotype leads to HIV persistence in treated patients, we identified an association between disease progression and several NK cell activating receptors. CD4/CD8 ratio has been previously implicated in T cell activation in vertical transmission but has not yet been shown in NK cells^[39]. Collectively, we established that most NK cell markers do not differ among our cohorts. This is unsurprising since most HIV-infected children had low viral loads and were on ART for several years. One caveat is that these results may be confounded by lack of adherence to medication regimens and coinfection status. Since ours is a small cross-sectional study, prospective multi-center longitudinal studies are needed to assess long-term variation in NK cell phenotype. At a minimum however, there are important signatures within the NK cell

populations of children that may provide insights into the underlying antiviral immune response and/or resistance to infection and disease progression.

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Figure legends

Fig 1. NK cell baseline frequency of most activating receptors in treated HIV-infected patients is elevated. NK cells were defined as CD3⁺CD45^{high}CD56⁺ using flow cytometry and gated above fluorescence minus one (FMO) controls for all markers. (A-F) Frequency of NK cells expressing DNAM-1, NKp30, NKG2C, CD16, CD11c, CD2 without stimulation. NKp46 and CD69 incubated in absence of stimulation (G, I) or after 4 hours stimulation with PMA/ionomycin (H, J). All markers indicate surface expression. Each data point represents a donor; Control (n=29) (red); HEU (n=23) (green); HIV-infected (n=25) (purple). All data shown is mean $\pm$ S.D. *p<0.05, **p<0.01, ***p<0.001 among three cohorts by Kruskal-Wallis and post-hoc comparison by Wilcoxon rank-sum test using Bonferroni adjustment.

Fig 2. Inhibitory receptor KIR2DL1 is found on a lower frequency of NK cells in treated HIV-infected patients. Frequency of NK cells with positive expression of KIR2DL1 but lacking CD158b, CD158e, KIR2DS4, CD94, NKG2A, NKG2C, KLRG1. Each data point represents a donor; HEU (n=23) (green); HIV-infected (n=25) (purple). Mean $\pm$ S.D. *p<0.05 using a general linear mixed model and adjusted for multiple comparisons using Holm's step-down Bonferroni method.

Fig 3. Frequency of NK cell activating and stimulatory receptors and CD57 negatively associates with CD4/CD8 T cell ratio. (A) Relationship between CD4/CD8 T cell ratio and frequency of NK cells expressing CD69 in the absence of stimulation (red) or increase in CD69 after stimulation with PMA/ionomycin (blue). (B-F) Relationship between CD4/CD8 T cell ratio and percentage of NK cells expressing KIR2DS4, CD244, NKG2C, CD11c and CD57. CD4/CD8 ratios were log transformed after a constant value of 1 was added to each ratio. All markers indicate surface expression. Each data point represents a donor; HIV-infected (n=24). P-values were identified by linear regression with the coefficient of determination (R^2) and slope defined.

Fig 4. Unstimulated NK cells in treated HIV-infected patients have higher degranulation potential and NK2 cytokine bias. IL-5 and CD107a incubated in absence of stimulation (A, C) or stimulated with PMA/ionomycin (B, D). Control (n=29) (red); HEU (n=23) (green); HIV-infected (n=25) (purple). All data shown is mean $\pm$ S.D. *p<0.05, **p<0.01, ***p<0.001 among three cohorts by Kruskal-Wallis and post-hoc comparison by Wilcoxon rank-sum test using Bonferroni adjustment. (E) Relationship between CD4/CD8 T cell ratio and frequency of NK cells expressing CD107a in the absence of stimulation (red) or increase in CD107a after stimulation (blue). (F) Relationship between CD4/CD8 T cell ratio and percentage of NK cells expressing granzyme in the absence of stimulation. CD107a indicates surface expression while granzyme and IL-5 were detected intracellularly as described in the Methods section. Each data point represents a donor; HIV-infected (n=24). P-values were identified by linear regression with the coefficient of determination (R^2) and slope defined.

Fig 5. NK cells in HIV-infected patients with asthma have increased frequency of IL-15 receptor- α chain, ICAM-1, KIR2DS4 but reduced inhibitory receptor KIR3DL1. (A-D) Frequency of NK cells expressing IL-15R α , CD54, KIR2DS4, CD158e among HIV-infected patients. No asthma (n=19) (black); Asthma (n=6) (red). KIR2DS4, No asthma (n=9). All data shown is mean $\pm$ S.D. *p<0.05, **p<0.01 by Wilcoxon rank-sum test.

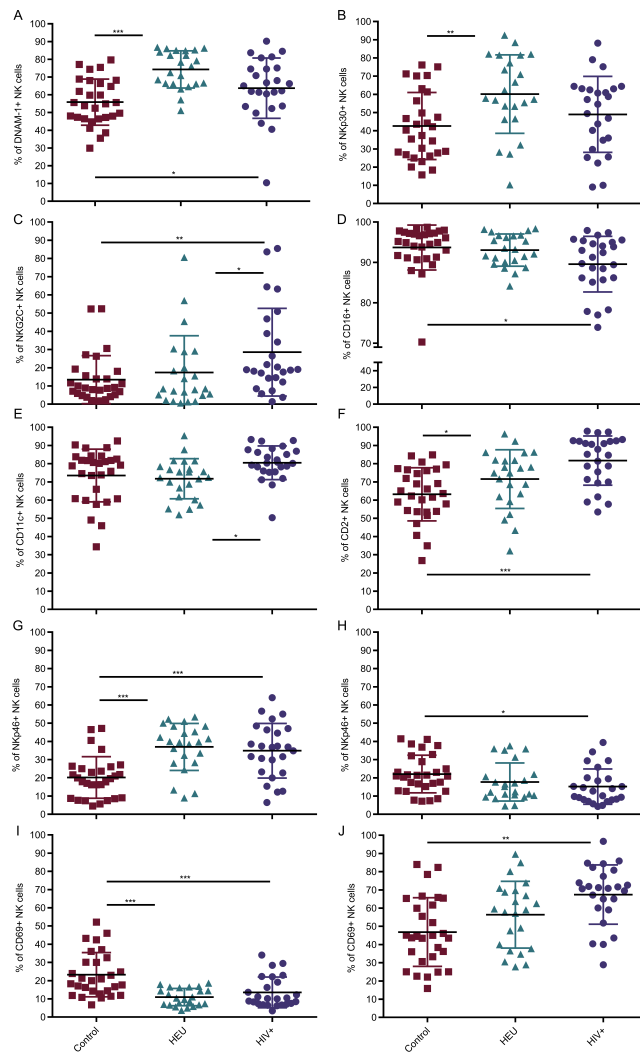
Fig 6. NK cell markers distinguishing HIV-infected, HEU and HIV-unexposed children. Frequency of NK cell markers that were statistically significant between one cohort and both other cohorts are indicated as being increased ($\uparrow$) or decreased ($\downarrow$). All other markers that did not differ between a pair of cohorts were considered similar as illustrated.

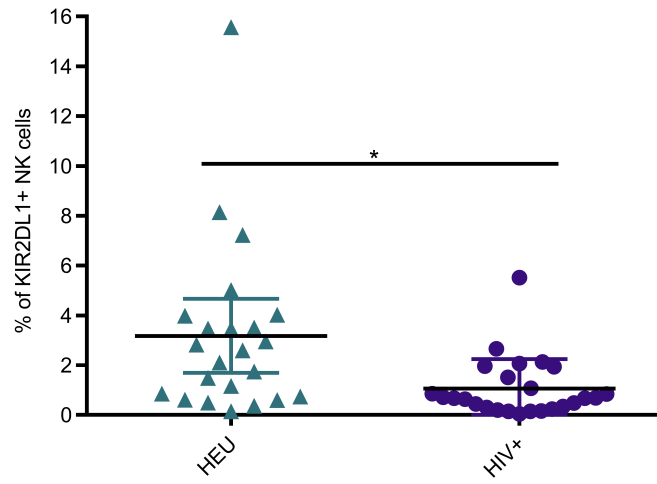
Table I. Study Cohort.

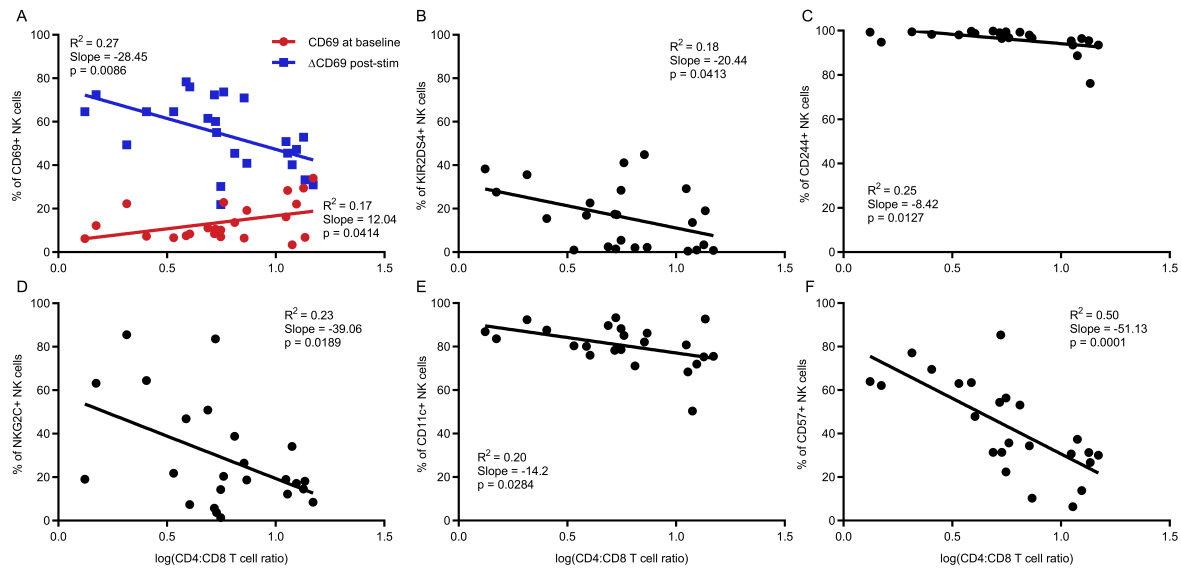
Age	Ethnicity	Sex	Cohort
3	Hispanic	F	HIV+
4	Unknown	F	HIV+
5	Black	F	HIV+
5	Black	F	HIV+
7	Black	F	HIV+
8	Black	F	HIV+
9	Hispanic	F	HIV+
11	Black	M	HIV+
12	Black	F	HIV+
12	Hispanic	F	HIV+
14	Black	F	HIV+
15	Hispanic	F	HIV+
15	Black	M	HIV+
16	Black	F	HIV+
16	Black	F	HIV+
16	Black	F	HIV+
17	Hispanic	M	HIV+
17	Black	M	HIV+
18	Black	F	HIV+
18	Black	M	HIV+
18	Black	F	HIV+
19	White (non-Hispanic)	M	HIV+
19	Black	F	HIV+
19	Black	M	HIV+
19	Black	M	HIV+

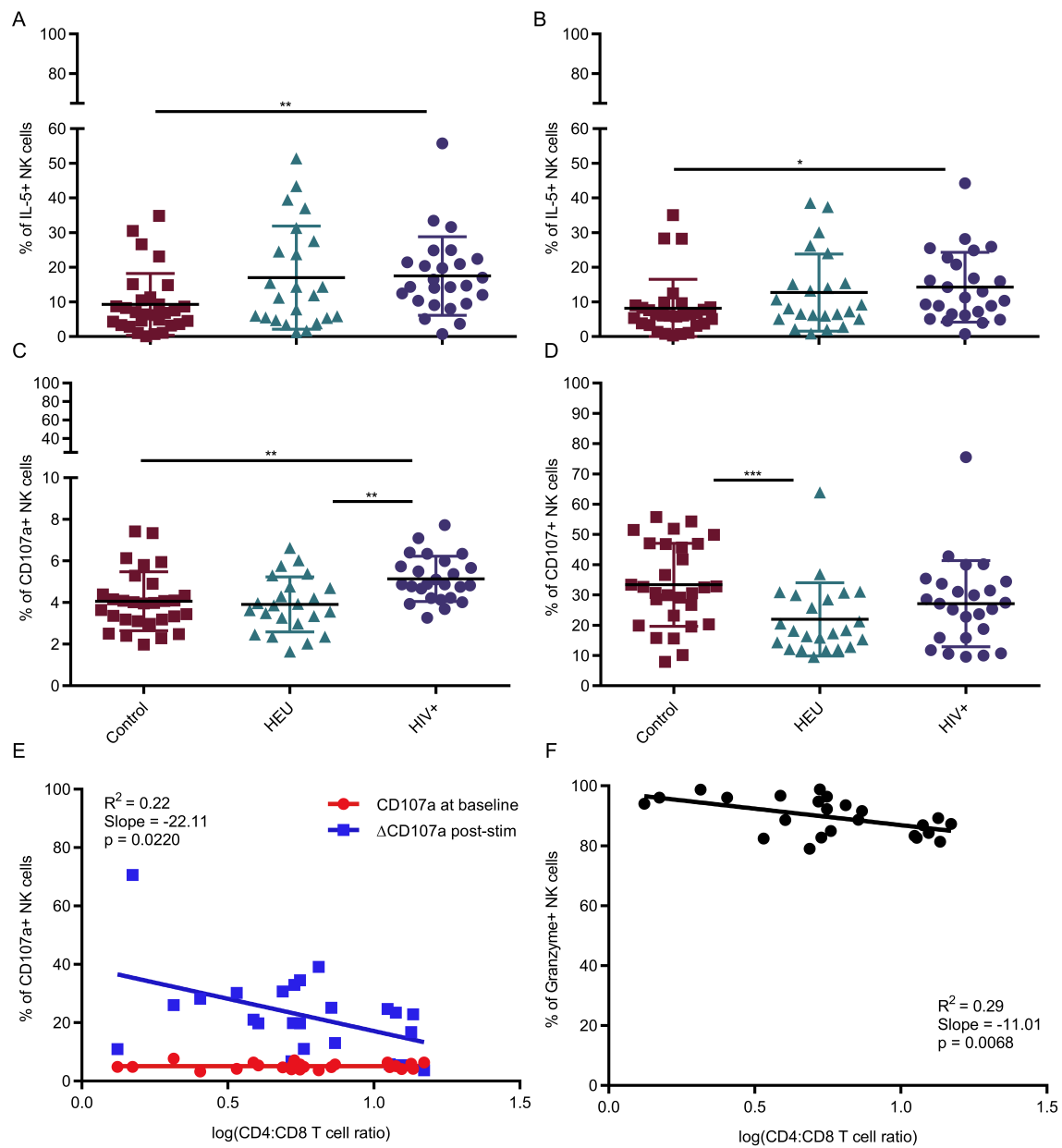
3	Hispanic	M	HEU
5	Black	F	HEU
5	Black	M	HEU
6	Black	F	HEU
7	Black	F	HEU
7	Black	F	HEU
8	Black	M	HEU
9	Black	M	HEU
9	White	M	HEU
10	Black	M	HEU
11	White (non-Hispanic)	M	HEU
11	Black	F	HEU
13	Black	M	HEU
14	Black	F	HEU
14	Black	M	HEU
15	Black	M	HEU
15	Black	M	HEU
15	Hispanic	M	HEU
16	Black	F	HEU
17	Black	F	HEU
18	Black	M	HEU
18	Black	F	HEU
19	Black	F	HEU
5	Hispanic	M	Control
6	Hispanic	M	Control
9	Hispanic	M	Control
9	Hispanic	F	Control

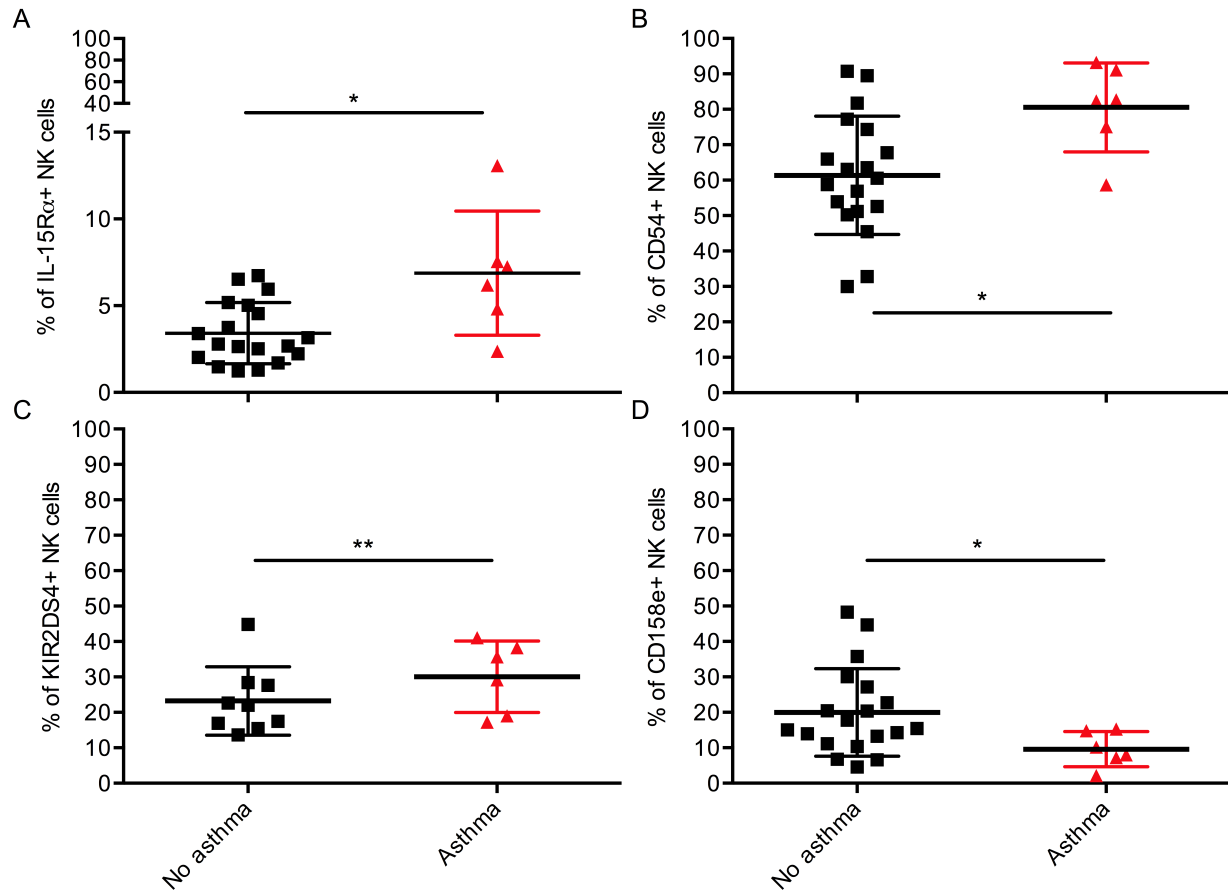
9	Black	M	Control
10	Black	M	Control
11	Black	F	Control
11	Hispanic	M	Control
12	Hispanic	M	Control
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13	Hispanic	M	Control
14	Hispanic	F	Control
14	Hispanic	M	Control
15	Hispanic	F	Control
15	Hispanic	F	Control
15	Black	F	Control
16	Unknown	M	Control
16	Hispanic	F	Control
16	Hispanic	M	Control
16	Hispanic	F	Control
16	Black	F	Control
17	Black	F	Control
18	Black	F	Control
18	Black	F	Control
18	Black	F	Control
18	Asian	M	Control
18	Hispanic	F	Control
19	Black	M	Control
19	White (non-Hispanic)	F	Control

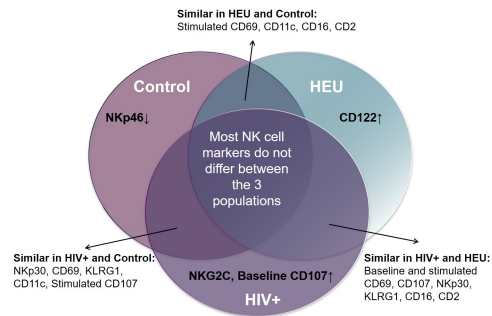


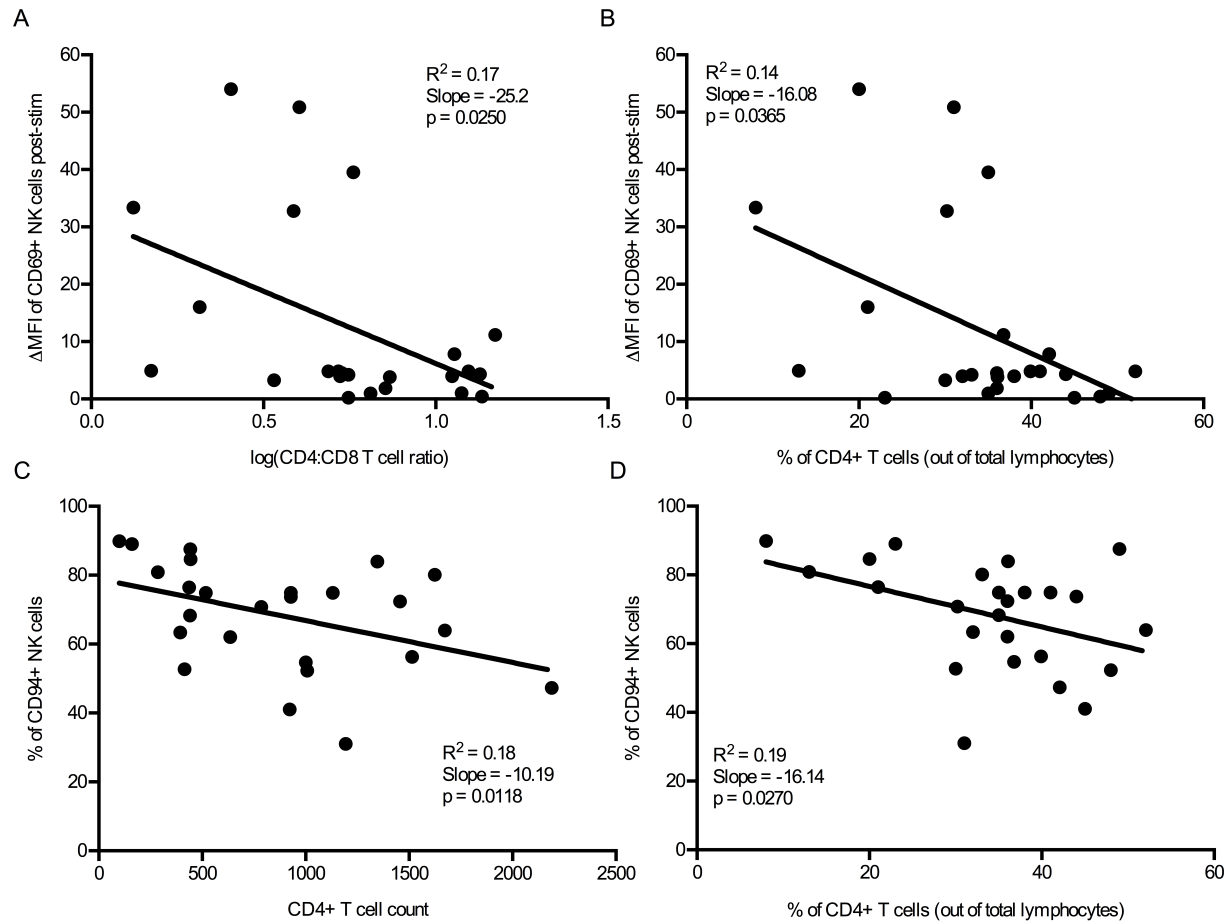


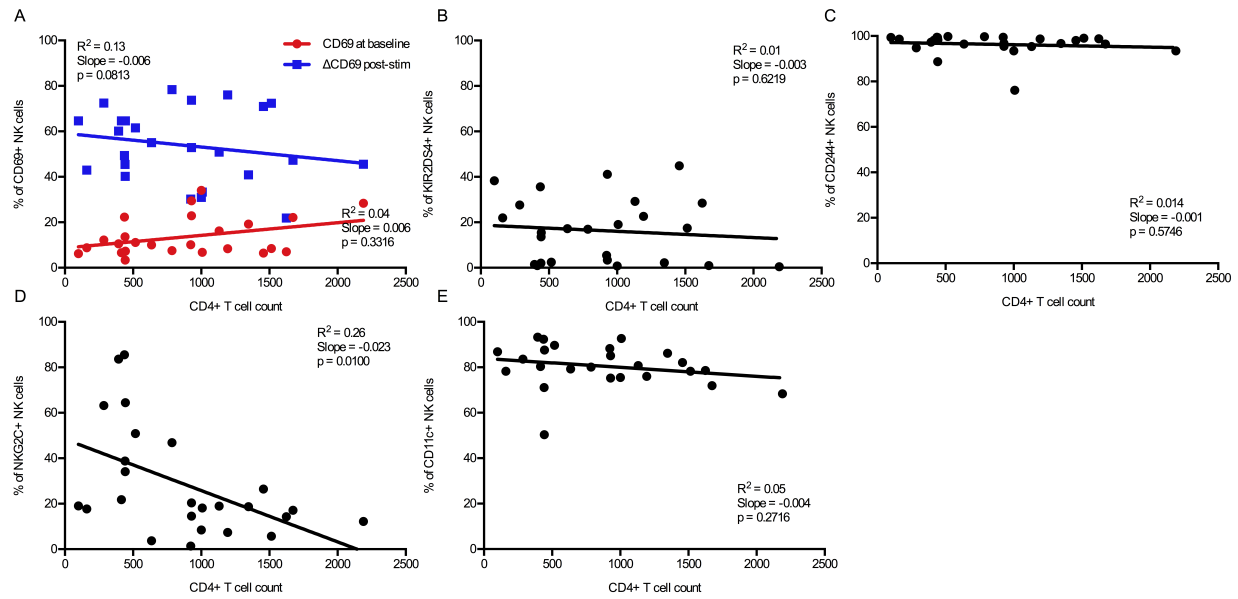


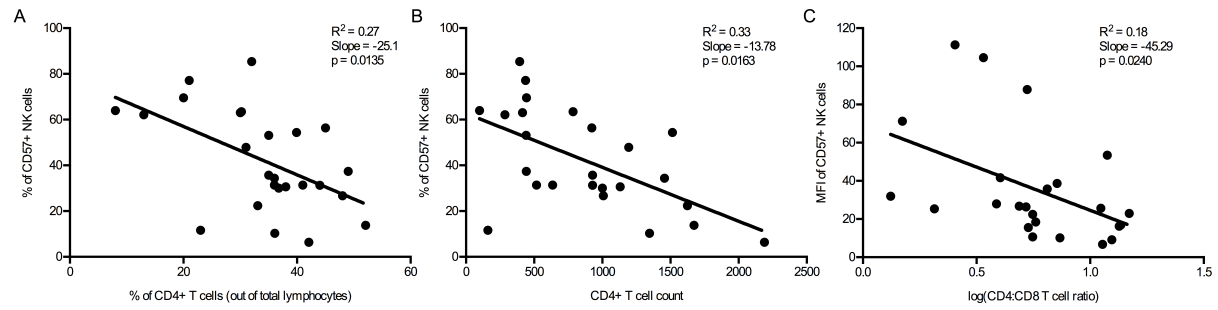




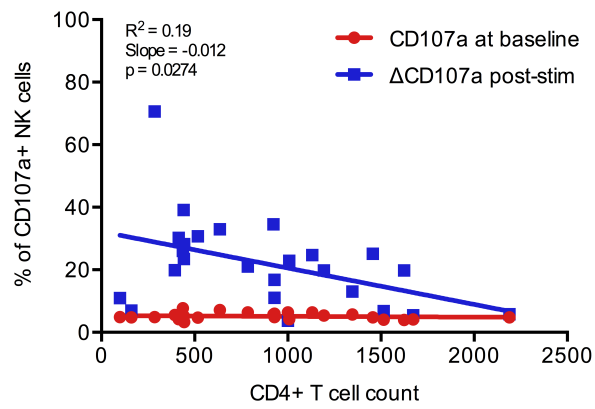




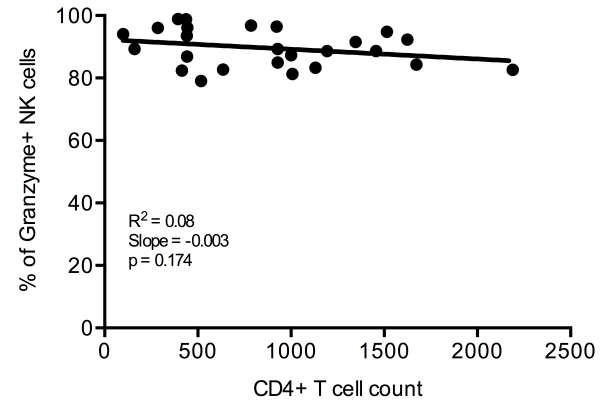


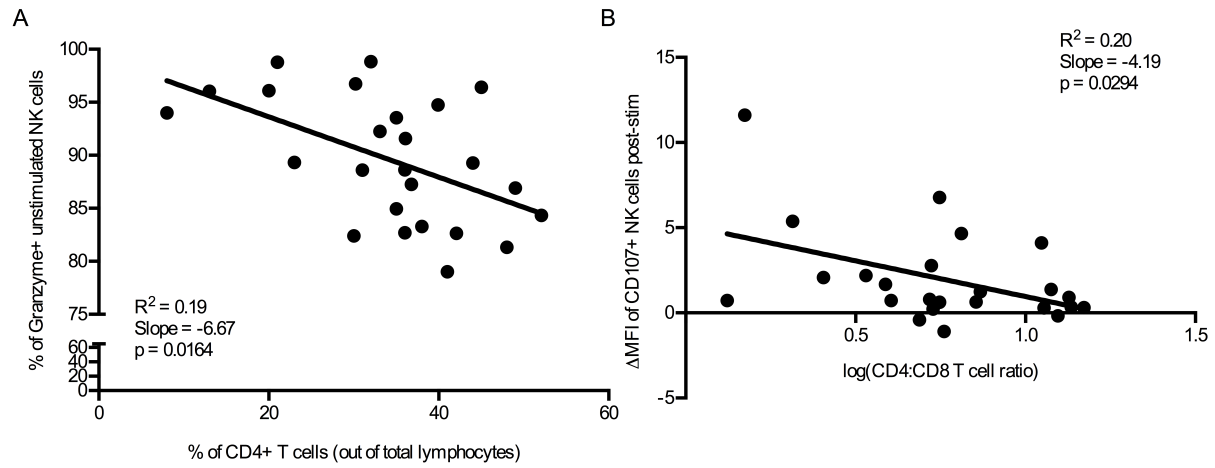


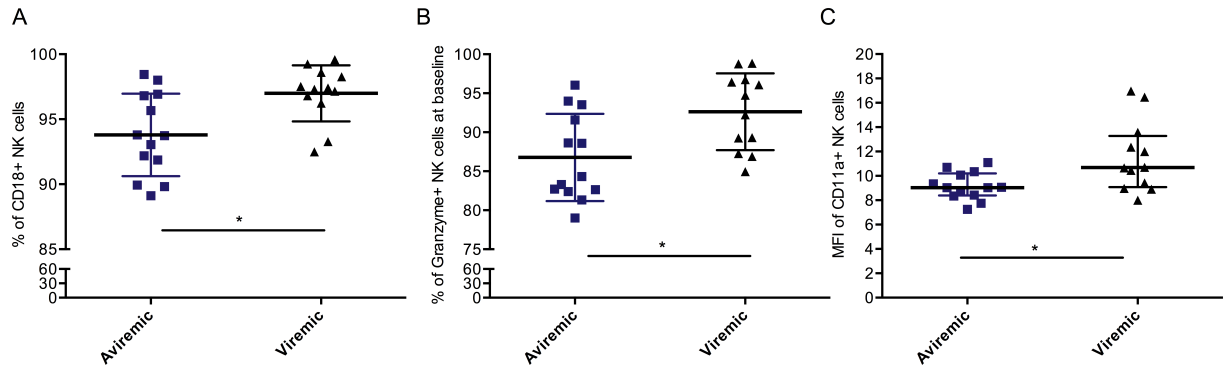
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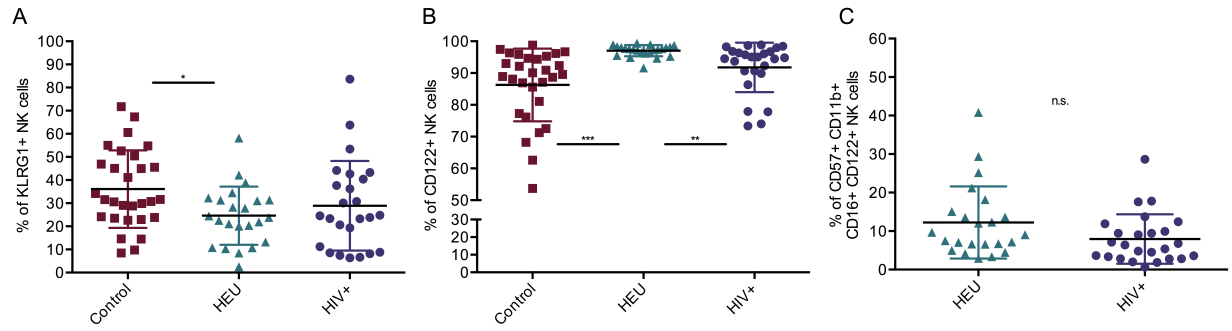


B









NK cells in treated HIV-infected children display altered
phenotype and function

Short title: NK cell phenotype in HIV-infected children

Sanjana MAHAPATRA^{a,b}, Ph.D., William T. SHEARER^{b,d}, M.D., Ph.D., Charles G. MINARD^c,
Ph.D., Emily MACE^{a,b,1}, Ph.D., Mary PAUL^d, M.D., and Jordan S. ORANGE^{a,b,d,1}, M.D., Ph.D.

^aDepartment of Pathology and Immunology, Baylor College of Medicine, Houston, Texas, United States of America;

^bCenter for Human Immunobiology, Texas Children's Hospital, Houston, Texas, United States of America;

^cDepartment of Medicine, Baylor College of Medicine, Houston, Texas, United States of America;

^dDepartment of Pediatrics, Baylor College of Medicine, Houston, Texas, United States of America;

¹Present address: Department of Pediatrics, Vagelos College of Physicians and Surgeon, Columbia University, New York, New York, United States of America

Corresponding author: Jordan S. Orange, CUMC/NewYork-Presbyterian Morgan Stanley Children's Hospital; 3959 Broadway, New York, NY 10032; E-mail: jso2121@cumc.columbia.edu; Tel: 212-305-2934; Fax: 212-305-3020

Supplementary captions

Fig S1. CD69 expression and frequency of CD94 on NK cells negatively associates with CD4/CD8 ratio and CD4+ T cells

Fig S2. Frequency of NKG2C negatively associates with CD4+ T cell count

Fig S3. CD57 has an inverse relationship with CD4+ T cells and CD4/CD8 ratio

Fig S4. Increase in CD107a frequency on stimulation negatively associates with CD4+ T cell count

Fig S5. Baseline granzyme and increase in CD107a on stimulation reciprocally associate with CD4+ T cells and CD4/CD8 ratio respectively

Fig S6. Viremic patients have increased adhesion receptors and baseline granzyme

Fig S7. NK cells in HEU children have highest CD122 and increased frequency of mature CD57+ subset

Table S1. NK cell phenotyping panels. *activating receptors; #inhibitory receptors (as appears in [14])

Table S2. Analysis of experimental variability in controls using Bland-Altman agreement study. (A) % bias after Bland-Altman analysis. (B) Statistical comparisons preserved after applying % bias to NK cell marker frequencies of each control

Table S3. HIV-infected cohort medication regimen and asthma history

Table S4. Top combinatorial subsets in HIV-infected and HEU pediatric patients. UD (undetectable); NRTI (nucleoside reverse transcriptase inhibitors); NNRTI (non-nucleoside reverse transcriptase inhibitors)

Table SI. NK cell phenotyping panels

Panel 1	Panel 2	Panel 3	Panel 4	Panel 5
Activating receptors	Adhesion/ stimulatory receptors	Inhibitory receptors	Developmental markers	Cytokines and effector molecules
CD45	CD45	CD45	CD45	CD45
CD56	CD56	CD56	CD56	CD56
CD3	CD3	CD3	CD3	CD3
CD8	CD16 [*]	CD158a/ KIR2DL1 [#]	IL-15R α	Perforin D48
DNAM-1 [*]	CD8	CD158b/ KIR2DL2-3 [#]	CD117/ c-kit	Perforin δ G9
NKG2D [*]	CD2 [*]	CD158e/ KIR3DL1 [#]	CD127	Granzyme B
NKp30/ NCR3 [*]	CD244/ 2B4 [*]	KIR2DS4 [*]	CD27	CD107a/ LAMP-1
NKp44/ NCR2 [*]	CD18	NKG2A [#]	CD62L	TNF α
NKp46/ NCR1 [*]	CD11a (LFA-1)	NKG2C [*]	CD94	IFN γ
CD69	CD11b (Mac-1, CR3)	CD94	CD122	IL-5
CD25	CD11c (CR4)	KLRG1 [#]	CD16	IL-13
	CD54/ ICAM-1		CD11b	IL-10
	CD28		CD57	

(as appears in [14]) ^{*}activating receptors; [#]inhibitory receptors

Table S2. Analysis of experimental variability in controls using Bland-Altman agreement study.

A.

	Stimulation	NK cell marker	Bias (%)	Control vs HIV+	Control vs HEU
Activating receptors	No stim	DNAM-1	9.7	No	No
		CD69	-3.02	No	Yes
		NKp30	2.97	N/A	Yes
		NKp46	2.35	Yes	Yes
		NKG2C	0.85	Yes	N/A
	With stim	NKp46	-2.35	No	N/A
		CD69	3.02	Yes	N/A
Adhesion/ stimulatory receptors		CD244	-1.22	No	N/A
		CD18	-2.97	No	No
		CD11b	-3.02	No	N/A
		CD11c	3.2	N/A	N/A
		CD2	1.63	Yes	No
Developmental markers		CD16	-0.68	Yes	N/A
		CD122	7.25	N/A	Yes
Cytokines and effector molecules	No stim	IL-5	7.25	No	N/A
		CD107a	4.97	Yes	N/A
	With stim	IL-5	7.25	No	N/A
		CD107a	-4.97	N/A	Yes

B.

Fluorochrome	Bias 1 (Panel 2)	Bias 2 (Panel 3)	Bias 3 (Panel 4)
BV 650	2.19		8.58
FITC	1.22	9.7	0.23
APC	2.97	2.74	1.56
APC Cy7		7.31	0.18

AF 700		8.9	
PE Cy7	0.02		0.19
PE Texas-Red	3.02		0.68
PerCP Cy5.5	3.2	10.79	
PE Cy5	4.97		1.72
PB	1.63	2.35	2.28
BV 785	0.72		0.86
PE	1.58	0.81	7.25

Table S3. HIV-infected cohort medication regimen and asthma history

Age	Viral load	CD4+ T cell counts	ART regimen			Asthma status
3	UD	1456	NRTI	Protease		None
4	79	1624	NRTI	Integrase		None
5	299	928	NRTI	Integrase		Asthma
5	UD	1347	NRTI	Integrase		None
7	UD	2189	NRTI	NNRTI		None
8	UD	1673	NRTI	Integrase		None
9	UD	1194	NRTI	Protease		None
11	556	1001	NRTI	Protease		None
12	UD	517	NRTI	Integrase		None
12	UD	1131	NRTI	Integrase		Asthma
14	23	1514	NRTI	Protease	Integrase	None
15	UD	1008	NRTI	Protease		Asthma
15	234	929	NRTI	Protease		None
16	4842	437	NRTI	Protease		Asthma
16	UD	635	NRTI	Integrase		Asthma
16	UD	414	NRTI	NNRTI		None
17	UD	286	NRTI	Protease		None
17	UD	441	NRTI	NNRTI		None
18	289	923	NRTI	Protease		None
18	48	442	NRTI	NNRTI		None
18	78	394	NRTI	Integrase		None
19	170	785	NRTI	NNRTI		None
19	UD	99	NRTI			Asthma
19	214	444	NRTI	Integrase		None
19	187	160	NRTI	Integrase		None

Table S4. Top combinatorial subsets in HIV-infected and HEU pediatric patients

Cell type	HEU		HIV+	
NK cells	DNAM-1-CD8-CD69-NKG2D-NKp30-NKp46-NKp44-CD25-	6.8	DNAM-1-CD8-CD69-NKG2D-NKp30-NKp46-NKp44-CD25-	10.1
CD56 ^{dim} NK cells	DNAM-1+CD8-CD69-NKG2D-NKp30-NKp46-NKp44-CD25-	12.5	DNAM-1+CD8-CD69-NKG2D-NKp30-NKp46-NKp44-CD25-	13.2
CD56 ^{bright} NK cells	DNAM-1+CD8-CD69-NKG2D-NKp30+NKp46+NKp44-CD25-	20.3	DNAM-1+CD8-CD69-NKG2D-NKp30+NKp46+NKp44-CD25-	16.7
NK cells	CD16 3G8+CD11a+CD11b+CD11c+CD18+ CD54+CD244+CD2+	26.7	CD16 3G8+CD11a+CD11b+CD11c+CD18+C D54+CD244+CD2+	34.9
CD56 ^{dim} NK cells		26.3		34.9
CD56 ^{bright} NK cells		29.2	CD16 3G8- CD11a+CD11b+CD11c+CD18+CD54+ CD244+CD2+	30.9
NK cells	KIR2DL1-CD158b-CD158e- KIR2DS4-CD94+NKG2A+NKG2C- KLRG1-	23	KIR2DL1-CD158b-CD158e-KIR2DS4- CD94+NKG2A+NKG2C-KLRG1-	20.8
CD56 ^{dim} NK cells		20.3		17.3
CD56 ^{bright} NK cells		46.9		49.2
NK cells	CD16 3G8+CD11b+ CD57 +CD94+CD62L- CD27-CD117-CD122+	16.9	CD16 3G8+CD11b+ CD57 - CD94+CD62L-CD27-CD117-CD122+	15.3
CD56 ^{dim} NK cells		17.8		16.3
CD56 ^{bright} NK cells	CD16 3G8+CD11b+CD57- CD94+CD62L+CD27+CD117- CD122+	22.9	CD16 3G8+CD11b+CD57- CD94+CD62L+CD27+CD117-CD122+	19.2
NK cells	Perf D48+Perf δG9+Granzyme+CD107a-IFNγ- TNFα-IL-5-IL-13-	65.6	Perf D48+Perf δG9+Granzyme+CD107a-IFNγ-TNFα- IL-5-IL-13-	62.6
CD56 ^{dim} NK cells		68.5		65.8
CD56 ^{bright} NK cells		32.3		32.2