Elevated Natural Killer Cell Activity Despite Altered Functional and Phenotypic Profile in Ugandans With HIV-1 Clade A or Clade D Infection

Michael A. Eller, MSc,†‡ Leigh Anne Eller, MSc,†‡ Benson J. Ouma, BBLT,† Doris Thelian, BS,‡ Veronica D. Gonzalez, MSc,* David Guwatudde, PhD,†§ Francine E. McCutchan, PhD,‡ Mary A. Marovich, MD,‡ Nelson L. Michael, MD, PhD,‡ Mark S. de Souza, PhD, MPH,‖ Fred Wabwire-Mangen, MD, PhD,†§ Merlin L. Robb, MD,‡ Jeffrey R. Currier, PhD,‡ and Johan K. Sandberg, PhD**

Background and Objective: Natural killer (NK) cells most likely contribute toward limiting HIV-1 replication, and investigation into their function throughout the course of infection is therefore important. We here aimed to determine the state of the NK cell compartment in Ugandans with untreated HIV-1 clade A or D infection in comparison with matched uninfected controls.

Methods and Results: The function and phenotype of NK cells were investigated using 10-color flow cytometry. Surprisingly, NK cells displayed elevated production of interferon-γ and macrophage inflammatory protein 1β, as well as CD107a degranulation in infected subjects. This included unexpected levels of degranulation in the CD56bright subset of NK cells and high levels of macrophage inflammatory protein 1β in CD56negative NK cells. HIV-1 infection was associated with reduced expression of KIR2DL1, NKG2A, CD161, and NKp30 in CD56dim and CD56negative NK cells, whereas lowered CD161 expression was the only alteration in the CD56bright subset. Interestingly, low CD4 counts were associated with increased levels of interferon-γ and degranulation in CD56bright NK cells, as well as increased NKp44 expression in the CD56dim cells.

Conclusions: NK cells in HIV-1–infected Ugandans display elevated activity, despite an altered functional and phenotypic profile. Furthermore, specific alterations in the CD56bright and CD56dim subsets occur in patients with severe CD4 loss.

Key Words: CD4, HIV-1, NK cells, viral infections

(*J Acquir Immune Defic Syndr* 2009;51:380–389)

INTRODUCTION

Natural killer (NK) cells are innate immune effector cells that play important roles in the control of certain types of tumors and microbial infections through cytotoxicity without prior sensitization, regulation of immune responses through cytokine, and chemokine production, as well as "cross talk" with antigen-presenting cells.[1,2] Human NK cells are commonly distinguished among peripheral blood lymphocytes based on expression of CD56 (neural cell adhesion molecule) and CD16 (FcγRIII) into several functionally and phenotypically distinct subsets.[1,3] NK cells are regulated by a vast array of stimulatory and inhibitory receptors that are designed for a non–antigen-specific recognition of infection and divided into 4 classes: killer immunoglobulin–like receptors (KIRs), C-type lectin receptors, natural cytotoxicity receptors (NCRs), and Toll-like receptors.[4,5] Using this receptor repertoire, NK cells are able to recognize and kill infected or malignant cells.[4,6] A major function of NK cells is the ability to lyse target cells through multiple mechanisms including exocytosis of cytotoxic granules, Fas ligand–mediated apoptosis, and antibody-dependent cellular cytotoxicity.[1,3] NK cells do, however, also exercise their diverse roles in immunity via the production of cytokines and chemokines.[1,7]

Acute HIV-1 infection has been associated with significant changes in NK cells, with a rapid expansion of cytolytic NK cells that is reduced after the emergence of HIV-specific CD8 T cells.[8] A different picture emerges during chronic infection where a skewing of NK subsets, with decreased CD56dim cytolytic cells and expanded CD56negative (CD56neg) NK cells, has been reported.[9–14] Chronic HIV-1 infection has been associated with a switch from inhibitory to activating C-type lectin receptor expression[15] and downregulation of NCRs.[11] NK cells suppress HIV-1

Received for publication January 17, 2009; accepted April 13, 2009.

From the *Center for Infection Medicine, Department of Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden; †Makerere University Walter Reed Project, Kampala, Uganda; ‡US Military HIV Research Program, Rockville, MD; §Makerere University School of Public Health, Kampala, Uganda; and ‖Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand.

Supported in part by the US Army Medical Research and Materiel Command and its Cooperative Agreement (W81XWH-04-02-0005) with the Henry M. Jackson Foundation for the Advancement of Military Medicine and with an Inter Agency Agreement (1Y-A1-26-42-07) with Division of Acquired Immunodeficiency Syndrome (DAIDS), National Institute of Allergy and Infectious Disease (NIAID), and National Institutes of Health (NIH). Additional work was supported in part by grants from the Swedish Research Council, the Swedish International Development Agency, the Swedish Foundation for Strategic Research, and the Swedish National Board of Health and Welfare.

Correspondence to: Michael A. Eller, MSc, Makerere University Walter Reed Project, Makerere University Medical School, Pathology Building A-10, Old Mulago Hill Rd, PO Box 16524, Kampala, Uganda (e-mail: meller@muwrp.org).

replication via direct cytotoxicity[16,17] and through the production of the CC-chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES, which can block virus entry through competitive inhibition of coreceptor binding.[5,18–20] Recent focus has been on KIRs, which determine NK cell reactivity via recognition of self-major histocompatibility complex (MHC) molecules. The activating receptor KIR3DS1, interacts with certain HLA-B alleles and is associated with delayed progression to AIDS,[21] decreased NK cell activation and increased function.[22] Furthermore, interactions between KIR and HLA molecules have been shown to directly influence the ability of NK cells to control HIV-1 replication in vitro.[23] KIR2DL3 expression is associated with NK cell activation and loss of function and inversely correlates to CD4 percentage in perinatally HIV-1–infected children.[24] There is also evidence that certain KIR and HLA interactions may affect the risk of HIV-1 transmission.[25] Together, these data suggest a profound impact of NK cells on the rate of HIV-1 disease progression.

The global HIV-1 epidemic continues to be a major medical concern in sub-Saharan Africa, where resources are least available to alleviate the effects of the infection. In Uganda, approximately 132,000 new HIV-1 infections occur annually with a national prevalence of 6.4%, and there is some evidence to suggest that this number is rising.[26] Despite improved access to antiretroviral therapy and increased focus on prevention, current interventions remain insufficient to cover estimated burden of HIV and AIDS in Uganda through 2010.[27] In addition, HIV-1 possesses extreme genetic diversity most strikingly in East Africa where subtypes A, C, and D and growing number of circulating recombinant forms are observed.[28,29] The viral subtype affects the rate of disease progression, specifically in Uganda where subtype D, recombinant infection, or multiple subtype infection is associated with more rapid progression to AIDS and death.[30] Evidence suggests decreased functionality in CD8 T cells and limited ability to control virus in clade D HIV-1 infection compared with clade A.[31]

To date, no studies have explored the involvement of innate cellular immunity in chronic HIV-1 infection in Uganda where subtypes A and D predominate. Such studies are needed to understand how NK cells contribute to control of infection and how the virus attempts to subvert the innate immune response. Recent methodological advances improve our ability to monitor the phenotype and function of NK cells.[32,33] In this study, we have investigated NK cells in untreated chronic HIV-1 infection in a cohort from Kayunga, a rural district in Uganda. We have analyzed the function and phenotype of NK cells and their subsets and investigated possible relationships with viral subtype, viral load, and disease progression. Interestingly, NK cells in infected subjects displayed elevated activity, despite an altered functional and phenotypic profile. Furthermore, certain alterations in the CD56bright and CD56dim subsets were found to occur specifically in patients with severe CD4 loss.

METHODS

Cohort

Study participants 15–49 years of age were enrolled in a prospective community-based cohort to assess the prevalence and incidence of HIV-1 infection in the Kayunga district, Uganda, in 2006 and 2007 (Table 1). HIV-1 testing was conducted at baseline, and both uninfected and infected volunteers were followed up every 6 months, with phlebotomy at each study visit. The study was approved by institutional review boards in both the US and Uganda.

Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from acid citrate dextrose-anticoagulated whole blood within 6 hours of collection by centrifugation through Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) using Leucosep tubes (Greiner Bio-One, Frickenhausen, Germany) at 800*g* for 15 minutes. The PBMC layer was harvested and washed with phosphate-buffered saline (PBS) 3 times by centrifugation at 250*g* for 10 minutes. Cell counts and viability were determined by hemacytometer using trypan blue exclusion. After PBMC count was determined, samples were centrifuged at 300*g* for 10 minutes, cryopreserved in freeze media (20% fetal bovine serum, 10% dimethyl sulfoxide, 1% Pen/Strep, and 69% 1640-RPMI), and stored in liquid nitrogen vapor.

TABLE 1. Study Population Descriptive Statistics

	HIV-1 Negative	HIV-1 Positive
Subjects, n	29	50
Sex, n (%)		
Female	17 (59)	29 (58)
Male	12 (41)	21 (42)
Median age (range), yrs	33 (19–48)	31 (19–48)
Immune phenotype		
CD4 absolute, median cells/mL (range)	973 (391–1444)	473 (12–1350)
CD4, median percent (range)	46 (28–54)	28 (1–43)
CD8 absolute, median cells/mL (range)	461 (171–800)	786 (331–2726)
CD8, median percent (range)	23 (7–34)	41 (21–71)
NK cell absolute, median cells/mL (range)	307 (66–1159)	219 (37–994)
NK cell, median percent (range)	13 (6–45)	11 (4–40)
B cell absolute, median cells/mL (range)	312 (140–649)	223 (79–572)
B cell, median percent (range)	16 (8–21)	13 (5–22)
Eosinophil, median percent (range)	10 (1–27)	11 (1–38)
Viral load, median copies/mL (range)	NA	47,967 (2702–>750,000)
HIV-1 subtype, n (%)		
HIV clade A	NA	30 (60)
HIV clade C	NA	1 (2)
HIV clade D	NA	19 (38)
Hepatitis B	4 (14)	2 (4)
Hepatitis C	1 (3)	1 (2)
Syphilis	1 (3)	5 (10)
HSV-2	14 (48)	43 (82)

NA, not available.

Serology

HIV-1 testing was performed on all participants as previously described.[34] Briefly, initial HIV-1 enzyme-linked immunosorbent assay (ELISA) testing was performed using the Genetic Systems rLAV (BioRad Laboratories, Redmond, WA) ELISA and confirmed with the Vironostika HIV-1 Microelisa Systems (Organon Teknika, Durham, NC) and the Genetic Systems HIV-1 Western Blot (BioRad Laboratories). HIV-1 viral load was determined for positive samples using the standard mode Amplicor HIV-1 Monitor test, version 1.5 (Roche Diagnostics, Indianapolis, IN).

Screening for hepatitis B surface antigen (HBsAg) was performed using Genetic Systems HBsAg EIA 3.0 (BioRad Laboratories) and confirmed using the Genetic Systems Confirmatory Assay 3.0 (BioRad Laboratories). Screening for antihepatitis C antibody was performed using the Ortho HCV version 3.0 ELISA Tests System and confirmed with the Chiron RIBA HCV 3.0 SIA (Chiron Corporation, Emeryville, CA). Syphilis serology was conducted using the Wampole Impact Rapid Plasma Reagin (RPR) kit (Inverness Medical Professional Diagnostics, Princeton, NJ) and confirmed using the Serodia *Treponemapallidum* particle agglutination (TP-PA) test (Fujirebio, Tokyo, Japan). All samples were tested for herpes simplex virus type 2 (HSV-2) antibody with an ELISA kit from Focus Diagnostics (Focus Technologies, Cypress, CA). A higher sample index value cutoff of 3.4 was used to optimize performance of the assay as previously described.[35]

HIV-1 Subtype Determination

All identified HIV-1 seropositive participants' plasma samples were tested for virus subtype using the previously described multiregion hybridization assay, version 2, for HIV-1 subtypes A, C, and D; recombinants; and dual infections.[28,36,37] HIV-1 subtype A, C, or D was assigned to 5 regions (*gag*, *pol*, *vpu*, *env*, and *gp41*) of the HIV-1 genome based on the reactivity of the probes, if at least 2 regions had probe reactivity. Samples with probe reactivity of a single subtype in different regions were labeled as pure subtypes.

Lymphocyte Immunophenotyping and Hematology

Lymphocyte immunophenotyping was performed on EDTA-anticoagulated whole blood using the FACS MultiSET System. Samples were run on a dual-laser flow cytometer using the single-platform multitest 4-color reagent in combination with TruCount tubes and analyzed using MultSET software (Becton Dickinson, San Jose, CA). The samples were used to determine absolute number and percentage of T-cell lymphocytes ($CD3^+$), helper T cell ($CD4^+$) and cytotoxic T cell ($CD8^+$), B cell ($CD19^+$), and NK cell ($CD16^+$ or $CD56^+$). Complete blood count with 5-part differential was performed on EDTA-anticoagulated whole blood using the Coulter AcT 5 diff (Beckman Coulter, Fullerton, CA).

NK Cell Distribution, Frequency, and Receptor Expression

Cryopreserved specimens were thawed and washed with complete media (CM): RPMI 1640 supplemented with 10% fetal bovine serum, 2% *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 2% l-glutamine, and 1% penicillin/streptomycin. Cells were distributed into 4 wells of a 96-well U-bottom plate and washed 3 times with PBS. Cells were stained with Aqua Live Dead Stain (Molecular Probes, Eugene, OR), washed 3 times with PBS, and blocked using Normal Mouse IgG (Caltag, Eugene, OR). Staining consisted of 4 different flow panels containing core monoclonal antibodies (mAbs) to differentiate NK cells and subsets and additional mAbs to assess activating and inhibitory receptor expression. The core panel distinguished NK cells based on expression of energy-coupled dye-conjugated anti-CD3 (Beckman Coulter), Alexa Fluor 700–conjugated anti-CD4, allophycocyanin (APC)-Cy7–conjugated anti-CD14 and anti-CD19, Pacific Blue–conjugated anti-CD16, and phycoerythrin (PE)-Cy7–conjugated anti-CD56 (BD Biosciences, San Jose, CA). The following mAbs were also added: panel 1, fluorescein isothiocyanate (FITC)-conjugated anti-KIR2DL1 (R&D Systems, Minneapolis, MN), PE-conjugated anti-KIR2DL2/3/DS2 (clone DX27) (BD Biosciences), and APC-conjugated anti-KIR2DL3 (R&D Systems); panel 2, FITC-conjugated anti-CD94 (BD Biosciences), PE-conjugated anti-NKG2C (R&D Systems), and APC-conjugated anti-NKG2A (R&D Systems); panel 3, FITC-conjugated anti-KIR3DL1 (clone DX9) (BD Biosciences), PE-conjugated anti-KIR3DL1/DS1 (clone z27) (Beckman Coulter), and APC-conjugated anti-CD161 (BD Biosciences); panel 4, PE-conjugated anti-NKp44 (Beckman Coulter) and Alexa Fluor 647–conjugated NKp30 (BD Biosciences). Cells were stained with the above mAbs for 30 minutes at 4°C, washed 3 times with PBS containing 0.5% bovine serum albumin and 0.1% sodium azide, and fixed in 2% formaldehyde (Tousimis Research, Rockville, MD) before data acquisition.

Assessment of NK Cell Function

PBMCs were thawed, washed with CM, and incubated for 18 hours with either the MHC^{null} K562 cell line (American Type Culture Collection) at an E:T ratio of 5:1 or the CM alone in a 96-well U-bottom plate in the presence of FITC-conjugated anti-CD107a (BD Biosciences), Brefeldin A (Sigma, St Louis, MO), and monensin (Becton Dickinson). After incubation, cells were washed with PBS; stained with Aqua Live Dead Stain; and blocked and stained for expression of CD3, CD4, CD19, CD14, CD16, and CD56. Cells were washed 3 times with PBS containing 0.5% bovine serum albumin and 0.1% sodium azide and fixed in 2% formaldehyde for 15 minutes at 4°C. Cells were permeabilized using Perm/Wash (Becton Dickinson); stained with energy-coupled dye-conjugated anti-CD3 (Beckman Coulter), PE-conjugated anti-MIP-1β, and FITC-conjugated anti-interferon (IFN)-γ for 30 minutes at 4°C; washed; and fixed in 2% formaldehyde.

Flow Cytometry

Cells were analyzed by flow cytometry using an LSRII instrument with 3 lasers (blue 488 nm, red 633 nm, and violet 405 nm) and 17 detectors (Becton Dickinson). Quality control SPHERO UltraRainbow Fluorescent Particles were run daily to maintain the instrument (Spherotech, Lake Forest, IL), and Simply Cellular antimouse compensation microspheres

(Bangs Laboratories, Fisher, IN) were used to complete the offline compensation matrix. In setting up the panels, matched isotype controls and "fluorescence minus one" samples were used to optimize the panels and set gates for analysis. Analysis was done using FlowJo Software version 8.5 (Tree Star, Ashland, OR).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism Software version 5.0a for Mac OSX (GraphPad Software, La Jolla, CA). Comparisons between groups were performed using Fisher exact test for categorical data. Direct comparisons between 2 groups were performed using the nonparametric Mann–Whitney *U* test for continuous data. Associations between NK cell function and phenotype against individuals' virological and immunological parameters were determined by Spearman rank test.

RESULTS

Impact of Untreated HIV-1 Infection on NK Cells and CD4 T Cells in a Rural East African Cohort

Groups of HIV-1–negative (n = 29) and HIV-1–positive (n = 50) individuals from a community-based cohort in Kayunga district, Uganda, were selected to assess the frequency, phenotype, and function of NK cells in chronic untreated HIV-1 clade A or D infection in comparison with uninfected matched controls (Table 1). Similar age and sex distribution was obtained in the 2 groups ($P = 1.000$, Fisher exact test). The occurrence of coinfections was similar in HIV-1–infected and HIV-1–uninfected subjects with regard to hepatitis B virus ($P = 0.185$), hepatitis C virus ($P = 1.000$), and syphilis ($P = 0.406$). However, HSV-2 infection was more common in the HIV-1–infected group, with 82% of HIV-infected subjects coinfected with HSV-2 and only 48% of HIV-uninfected subjects infected with this virus ($P = 0.001$). Lymphocyte immunophenotyping was performed on whole blood to determine the absolute number and percentage of lymphocyte subsets. As expected, HIV-1–infected individuals had lower CD4 counts compared with uninfected controls ($P < 0.001$), whereas no difference was observed in the overall NK cells, although there was a trend toward reduced NK cell numbers ($P = 0.105$; Fig. 1A). Infected subjects had lower B-cell absolute counts ($P = 0.011$) and increased CD8 T-cell absolute counts ($P < 0.001$), whereas there was no difference in the overall CD3 cell counts (data not shown). As expected, CD4 T-cell counts correlated inversely with plasma viral load ($P = 0.007$, $r = -0.379$; Fig. 1B). Interestingly, although NK cell counts showed no correlation with viral load, they did correlate directly with CD4 T-cell counts ($P = 0.035$, $r = 0.291$; Figs. 1C, D). There was no difference in the overall immune phenotype between HIV-1 clade A and clade D infection (data not shown). The correlation between NK cell counts and CD4 counts suggests a coordinated insult to these 2 lymphocyte compartments.

Altered NK Cell Subset Distribution in HIV-Infected Ugandans

NK cells are divided into subsets depending on their CD56 expression level, and these subsets are phenotypically and functionally distinct.[1,3] We therefore investigated in detail the CD56bright, CD56dim, and CD56neg subsets of NK cells in HIV-1–infected and HIV-1–uninfected Ugandans. Multicolor flow cytometry was used to distinguish NK cells based on lack of CD3, CD4, CD14, and CD19 expression to exclude T cells, monocytes, and B cells. NK cell subsets were

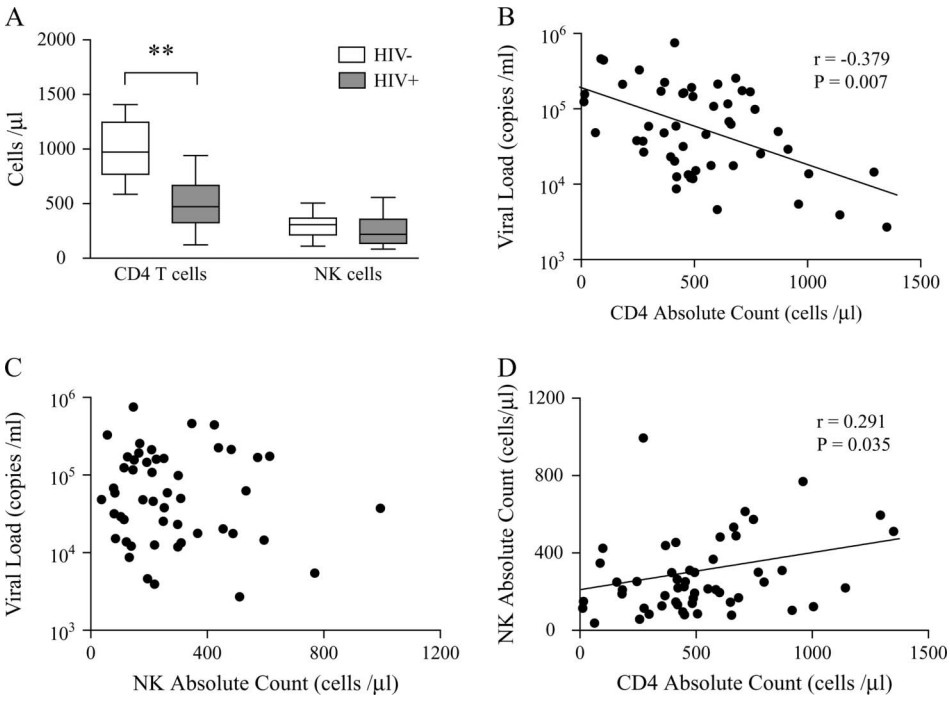

FIGURE 1. Immune phenotype in HIV-1–infected and HIV-1–uninfected Ugandans and the relationship to plasma viral load. A, Absolute counts of CD4 T cells but not of NK cells are significantly reduced in chronic HIV-1 infection. B, CD4 T-cell absolute counts correlate with plasma viral load ($\log_{10}$) copies per milliliter ($P = 0.007$, $r = -0.379$). C, Absolute NK cell counts do not correlate to plasma viral load ($P = 0.644$, $r = 0.067$). D, NK cell absolute counts correlate with CD4 T-cell absolute counts ($P = 0.035$, $r = 0.291$). **P is significant 0.01 to 0.05.

identified in this population based on expression of CD16 and/or CD56 (Fig. 2B). Examination of the 3 subsets revealed comparable levels of CD56bright cells in HIV-1–infected and HIV-1–uninfected subjects: 3.9% and 2.7%, respectively. However, CD56dim NK cells were reduced from 88.5% to 65.8% in HIV-1 infection ($P < 0.001$), and CD56neg cells were increased from 5.1% to 25.8% ($P < 0.001$; Fig. 2B). Subset distribution was similar in HIV-1 clade A–infected subjects and HIV-1 clade D–infected subjects (data not shown). Taken together, these data indicate that chronic untreated HIV-1 infection in rural Uganda leads to a skewing among the functionally distinct CD56dim and CD56neg subsets.

Altered Expression of Activating and Inhibitory NK Cell Receptors in HIV-1 Clade A and D Infection

NK cell activity is regulated by a vast array of activating and inhibitory receptors, and we next assessed expression of a panel of such receptors in HIV-1–infected and HIV-1–uninfected subjects. Reduced expression of KIR2DL1 ($P = 0.005$), NKG2A ($P = 0.027$), CD161 ($P = 0.003$), and NKp30 ($P = 0.002$) was observed in the overall NK cell compartment in HIV-1–infected individuals compared with HIV-1–uninfected subjects (Fig. 2C). Within the CD56bright NK subset, a significant reduction was observed in CD161 expression ($P = 0.003$), whereas a small increase was observed in KIR3DL1/DS1 (z27) in infected subjects ($P = 0.028$; Fig. 2D). In the CD56dim cells, a significant reduction was observed in the expression of KIR2DL1 ($P = 0.005$), CD161 ($P = 0.013$), and NKp30 ($P = 0.017$). In addition, a significant increase was seen in NKp44, although expression levels remained very low ($P = 0.044$; Fig. 2E). Finally, the CD56neg subset had reduced expression of KIR2DL1 ($P = 0.004$), NKG2A ($P = 0.040$), and NKp30 ($P = 0.039$), and an increase in NKp44 expression ($P = 0.022$), in infected subjects compared with uninfected controls (Fig. 2F). In a comparison of the 3 subsets, the CD56bright subset stood out with little KIR expression and high NKp30 and CD94/NKG2A expression that were not reduced by HIV-1 infection. The CD56dim and CD56neg subsets were relatively similar, although levels of activating receptors were generally lower in the CD56neg cells. No significant differences in NK receptor expression were noted when clade A–infected subjects and clade D–infected subjects were compared (data not shown). Together, these data indicate that HIV-1 infection in this cohort results in reduced expression of certain activating and inhibitory receptors in NK cells, but the extent of these changes varies among the 3 NK cell subsets.

Enhanced NK Cell Activity and Skewed Functional Profile in HIV-1–Infected Subjects

We compared the functional activity of NK cells in the healthy and HIV-1–infected groups. Thawed PBMC samples were either washed and stimulated overnight with the MHC^{null} K562 cell line at an effector to target ratio of 5:1 or cultured in the absence of exogenous stimulation. The activity was measured by the ability to degranulate using the CD107a assay,[32] and by the production of IFN-γ and MIP-1β, to ascertain the functional profile of NK cells (Fig. 3A). The 3 subsets displayed different functional profiles in uninfected subjects (Fig. 3B). The CD56bright NK cells were the least responsive with little IFN-γ and MIP-1β production, although degranulation was comparable to the CD56dim subset. CD56dim cells had significantly higher IFN-γ expression compared with the other 2 subsets and also produced high levels of MIP-1β. Finally, the CD56neg cells had only low levels of degranulation and IFN-γ expression, whereas they expressed high levels of MIP-1β.

In HIV-1–infected subjects, the total NK cell population showed increased spontaneous activity with regard to CD107a ($P < 0.001$), IFN-γ ($P = 0.034$), and MIP-1β ($P < 0.001$), whereas only CD107a expression was increased in the K562-stimulated samples compared with uninfected controls ($P = 0.014$; Fig. 3C). In the NK cell subset analysis, HIV-1–infected subjects' CD56bright NK cells showed increased spontaneous expression of CD107a ($P = 0.011$), whereas both CD107a ($P < 0.001$) and IFN-γ ($P = 0.003$) were increased in this subset in the K562-stimulated samples (Fig. 3D). The CD56dim population of NK cells showed increased spontaneous production of CD107a ($P < 0.001$), IFN-γ ($P = 0.005$), and MIP-1β ($P < 0.001$) in HIV-1–infected subjects, whereas both CD107a ($P = 0.001$) and MIP-1β ($P = 0.019$) were increased in this subset after K562 stimulation (Fig. 3E). Finally, CD56neg NK cells showed increased expression of CD107a ($P < 0.001$), IFN-γ ($P = 0.004$), and MIP-1β ($P < 0.001$) in the unstimulated samples, whereas CD107a ($P < 0.001$) and MIP-1β ($P = 0.002$) were increased in the K562-stimulated samples from HIV-1–infected subjects (Fig. 3F). In addition, looking exclusively at the K562-stimulated response in HIV-infected subjects, there was a marked decrease in CD107a ($P = 0.005$) and IFN-γ ($P < 0.001$) and a trend toward increased MIP-1β production in the CD56neg subset compared with the CD56dim NK subset (data not shown). A similar pattern was observed in the HIV-1–uninfected individuals (Fig. 3B). Taken together, these data imply that there is no broad dysfunction in the NK cell compartment with regard to the functional aspects we have investigated. In fact, the spontaneous activity measured without additional in vitro stimulation suggests that the activity of NK cells may be elevated in subjects with chronic untreated HIV-1 clade A and D infection.

CD56bright and CD56dim NK Cell Function and Phenotype Relate to Disease Progression

The present datasets were next analyzed for possible correlations between the NK cell phenotype and function and markers of HIV-1 disease progression. Evaluation of phenotypic alterations observed in HIV-1 infection showed an inverse correlation between NKp44 expression in CD56dim NK cells and the CD4 T-cell absolute count ($P = 0.002$, $r = -0.425$; Fig. 4A). Furthermore, there was an inverse correlation between CD56bright NK cell expression of KIR3DL1/DS1 (clone z27) and CD4 absolute count ($P = 0.050$, $r = -0.279$; Fig. 4B). When the differences in NK cell function were analyzed in relation to viral load and CD4 T-cell decline, there was no correlation with overall NK cell activity,

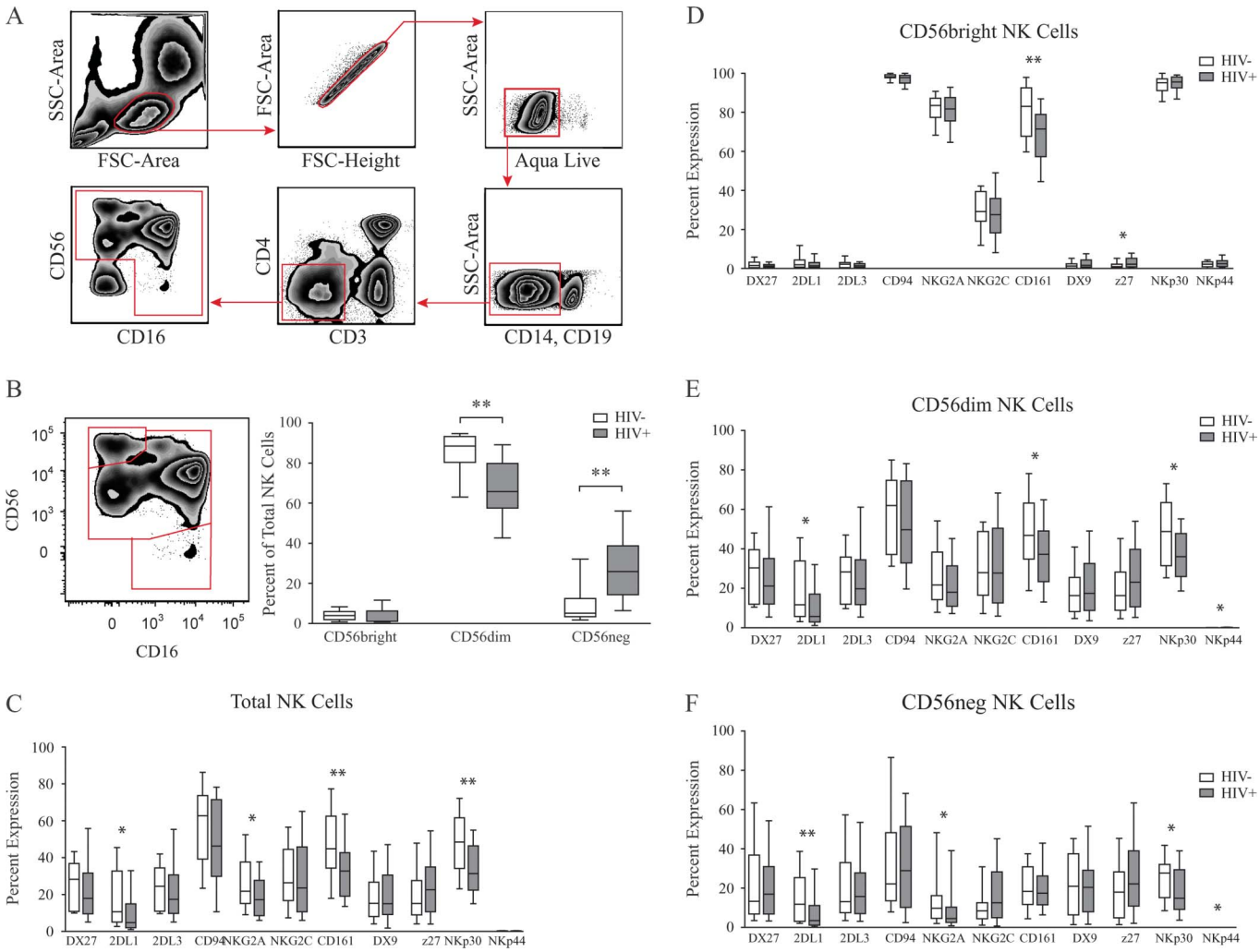

FIGURE 2. NK cell subset distribution and phenotype. Cryopreserved PBMCs were thawed and stained with mAbs against CD3, CD4, CD14, CD16, CD19, and CD56 to determine the NK cell percentage and distribution of subsets. A, Zebra plots illustrating the gating strategy for identification of NK cells. B, Zebra plot illustrating identification for NK cells subsets and box and whisker plots showing the median and 10th–90th percentiles of NK cell subsets in HIV-1–infected and HIV-1–uninfected subjects. CD56dim NK cells are significantly reduced in chronic HIV-1 infection and CD56neg cells are increased as determined using the Mann–Whitney *U* test, both 2-tailed $P < 0.001$. C, Activating and inhibitory receptor expression on NK cell subsets changes in chronic HIV-1 infection. Expression of the following receptors was assessed by flow cytometry: KIR2DL1, KIR2DL2/3/DS2 (clone DX27), KIR2DL3, CD94, NKG2A, NKG2C, KIR3DL1 (DX9), KIR3DL1/DS1 (z27), CD161, NKp30, and NKp44. Box and whisker plots showing the median and 10th–90th percentiles of receptor expression in the total NK cell population in HIV-1–infected and HIV-1–uninfected study participants. Statistically significant difference between the groups was observed for KIR2DL1 ($P = 0.005$), NKG2A ($P = 0.027$), CD161 ($P = 0.003$), and NKp30 ($P = 0.002$) expression using the Mann–Whitney *U* test. D, Box and whisker plots of CD56bright NK cell receptor expression. Statistically significant difference between the study groups was observed for CD161 ($P = 0.003$) and KIR3DL1/DS1 (z27) ($P = 0.028$) expression using the Mann–Whitney *U* test. E, Box and whisker plots showing receptor expression in CD56dim NK cells. Statistically significant difference between the groups was observed for KIR2DL1 ($P = 0.005$), CD161 ($P = 0.013$), NKp30 ($P = 0.017$), and NKp44 ($P = 0.044$) expression using the Mann–Whitney *U* test. F, Box and whisker plots showing receptor expression in CD56neg NK cells. Significant differences between the groups were observed for KIR2DL1 ($P = 0.004$), NKG2A ($P = 0.034$), NKp30 ($P = 0.039$), and NKp44 ($P = 0.022$) expression using the Mann–Whitney *U* test. *P is significant 0.01 to 0.05; **P is very significant <0.01.

CD56dim NK cell activity, or CD56neg NK cell activity (data not shown). Interestingly, however, an inverse correlation was observed between IFN-γ expression in CD56bright NK cells and CD4 absolute counts ($P = 0.019$, $r = -0.335$; Fig. 4C). An inverse correlation was also observed between the K562-induced degranulation in CD56bright NK cells and CD4 absolute counts ($P = 0.003$, $r = -0.423$; Fig. 4D). These data suggest a relationship between the functional and phenotypical status of CD56bright and CD56dim NK cells and HIV-1 disease progression.

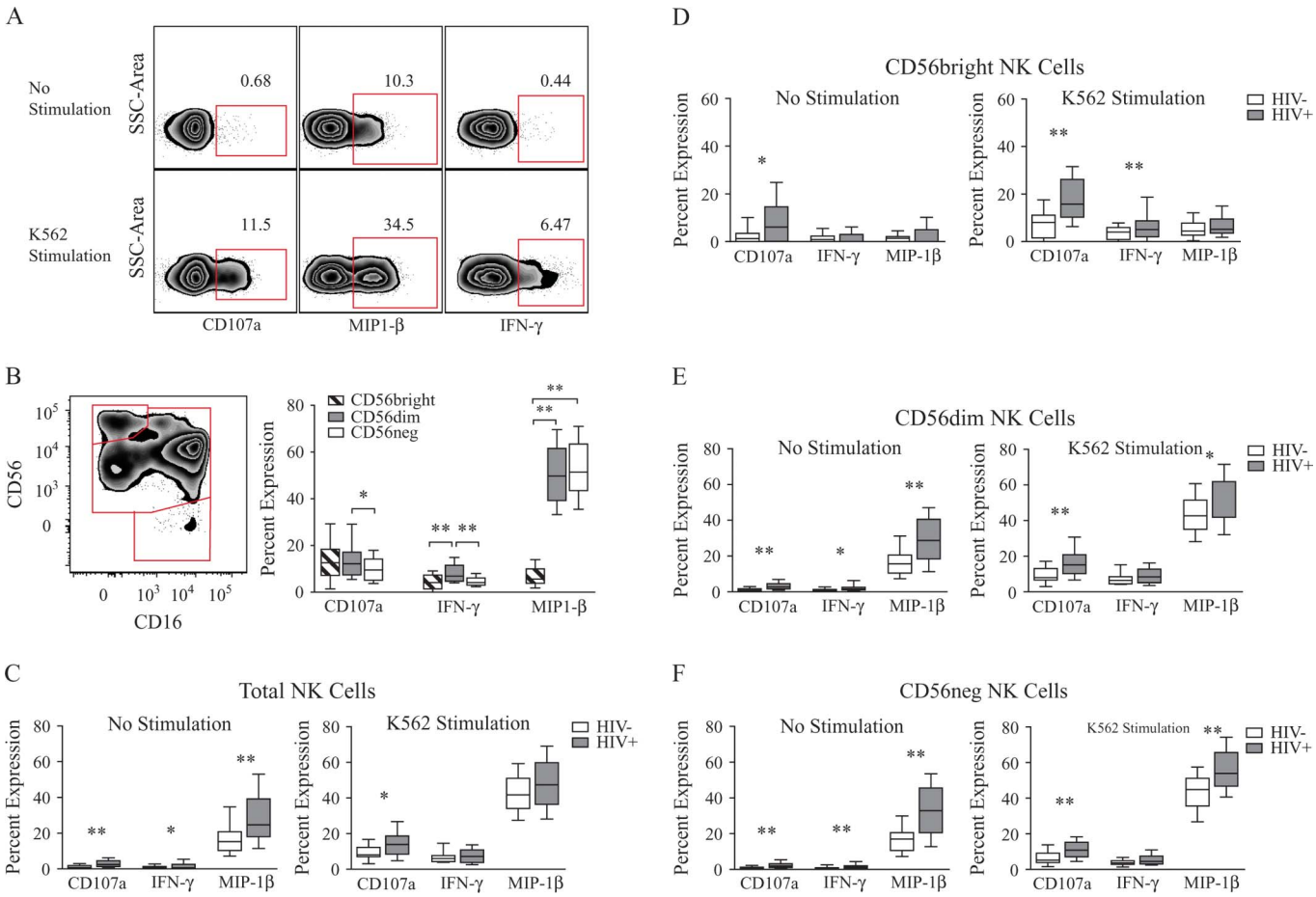

FIGURE 3. Changes in NK cell function in HIV-1 infection. Cryopreserved PBMCs were thawed and cultured overnight in the presence of mAb against CD107a and presence or absence of MHC^{null} K562 cells. After overnight incubation, cells were stained extracellularly for expression of CD4, CD19, CD14, CD16, and CD56 and intracellularly for CD3, IFN-γ, and MIP-1β to determine function. A, Representative zebra plots showing unstimulated and K562-stimulated CD107a, IFN-γ, and MIP-1β expression. B, Zebra plot illustrating identification of NK cell subsets and box and whisker plot showing the median and 10th–90th percentiles of the NK cell subsets' functional ability in healthy subjects. CD107a is reduced in CD56neg NK cells compared with CD56dim (P = 0.014). IFN-γ is increased in CD56dim NK cells compared with CD56bright and CD56neg NK subsets (both $P < 0.001$). MIP-1β production is increased in both CD56dim and CD56neg subsets compared with CD56bright (both $P < 0.001$). C, Box and whisker plots showing the median and 10th–90th percentile frequency of the total NK cell population expressing CD107a, IFN-γ, and MIP-1β in unstimulated (left panel) and K562-stimulated (right panel) cultures. HIV-1 infection was associated with higher expression of CD107a ($P < 0.001$), IFN-γ ($P = 0.034$), and MIP-1β ($P < 0.001$) in the unstimulated cultures, whereas CD107a ($P = 0.014$) was increased in the K562-stimulated samples. D, Box and whisker plots showing responses in CD56bright NK cells. HIV-1 infection was associated with higher expression of CD107a ($P = 0.011$) in the unstimulated cultures, whereas CD107a ($P < 0.001$) and IFN-γ (P = 0.003) were increased in the K562-stimulated samples. E, Box and whisker plots showing responses in CD56dim NK cells. HIV-1 infection was associated with higher expression of CD107a ($P < 0.001$), IFN-γ ($P = 0.005$), and MIP-1β ($P < 0.001$) in the unstimulated cultures, whereas CD107a ($P = 0.001$) and MIP-1β ($P = 0.019$) were increased in the K562-stimulated cells. F, Box and whisker plots showing CD56neg NK cells. HIV-1 infection was associated with increased expression of CD107a ($P < 0.001$), IFN-γ ($P = 0.004$), and MIP-1β ($P < 0.001$) in the unstimulated cells, and CD107a ($P < 0.001$) and MIP-1β ($P = 0.002$) were increased in the K562-stimulated cells. *P is significant 0.01 to 0.05; **P is very significant <0.01.

DISCUSSION

NK cells most likely contribute to control of HIV-1 viremia throughout the course of infection via their cytotoxic function[16,17] and production of cytokines and CC-chemokines.[5,18–20] Still, however, much remains to be learned about how NK cells work to help control HIV-1 infection, the means of viral evasion from these cells, and how chronic infection affects NK cell function. The need for more studies is particularly pressing in locations where great viral diversity is encountered, such as rural Africa. We have characterized the frequency, phenotype, and function of NK cells and their subsets in patients with chronic untreated HIV-1 clade A and clade D infection in the rural district of Kayunga, Uganda. We have found that NK cells maintain a high or even elevated

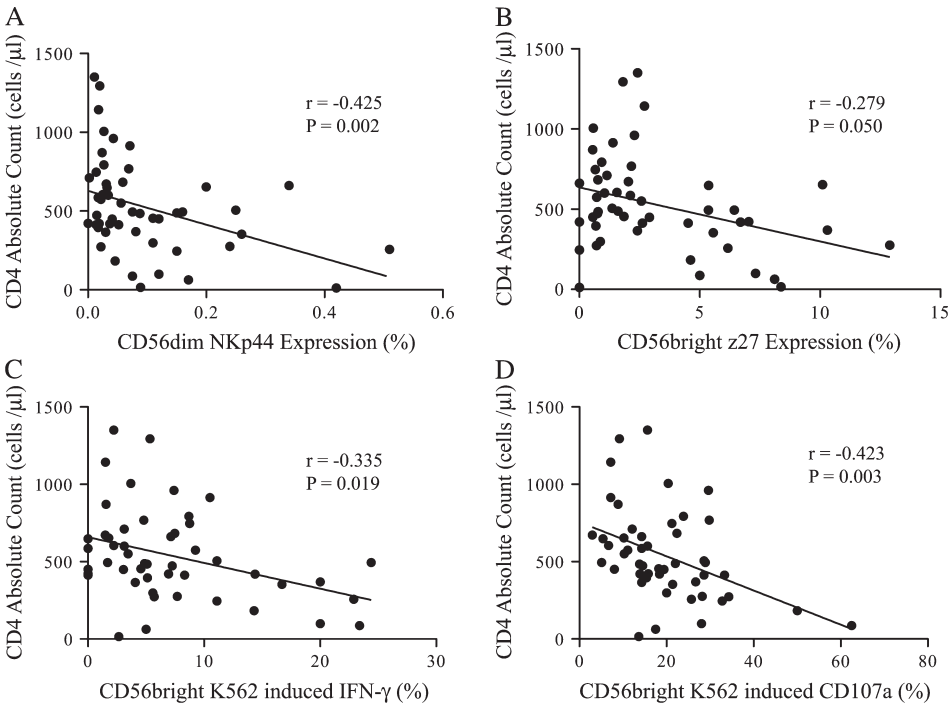

FIGURE 4. Changes in NK cell phenotype and function correlate with CD4 loss. Phenotypic assessment of the expression of surface receptors on NK cells was compared with CD4 T-cell absolute counts. A, Inverse correlation between NKp44 expression in CD56dim NK cells and CD4 T-cell absolute count (P = 0.002, r = −0.425). B, Inverse correlation between CD56bright NK cell expression of the mAb clone z27 epitope and CD4 T-cell absolute count (P = 0.050, r = −0.279). Functional assessment of NK cell subsets after culture with the MHC^{null} K562 cell line, and responses were compared with CD4 absolute counts. C, Inverse correlation between CD56bright NK cell expression of IFN-γ and the CD4 absolute counts (P = 0.019, r = −0.335). D, Inverse correlation between CD56bright NK cell expression of CD107a and CD4 absolute counts (P = 0.003, r = −0.423).

activity in this setting, despite altered NK cell receptor expression and a skewed subset distribution and function. Furthermore, we have identified correlates between the functional and phenotypical status of CD56bright and CD56dim NK cells and CD4 counts.

The effect of persistent HIV-1 replication during chronic infection has been associated with immune dysregulation that is not clearly understood in NK cells. We found that despite the CD4 T-cell loss associated with HIV-1 disease, the median NK cell absolute counts remained essentially normal. In contrast, the NK cell subset distribution was altered with a skewing toward increased CD56neg NK cells and decreased CD56dim NK cells. Altered NK cell subset distribution is consistent with previously published studies of cohorts in Europe and America showing a general increase in the CD56neg NK cell subset proposed to represent dysfunctional NK cells,[5,12,14] with relatively little change to the overall absolute NK cells numbers. Furthermore, we observed that the NK cell absolute numbers were directly associated with CD4 absolute counts but not viral load, suggesting that NK cell decline with progressive disease may be secondary to virus-driven CD4 T-cell loss. It is important to note that no difference was observed between HIV-1 clade A–infected subjects and HIV-1 clade D–infected subjects with regard to the NK cell numbers, subset distribution, or function during chronic HIV-1 infection. There is some evidence that NK cells may exert the most influence on immune regulation and disease progression during the acute stage of infection.[8,12] Examining acute infection time points could potentially unveil aspects of NK cells in HIV-1 clade D infection, which contribute to the more rapid progression to AIDS associated with this viral subtype.

The function of NK cells has been well characterized with regard to the ability of these cells to lyse infected or malignant cells and produce cytokine and chemokines. The distribution of these activities in the NK cell subsets is less clear. In this study, we found that uninfected Ugandans have a similar distribution of NK cell subsets as previously reported in studies from industrialized countries.[1,3,5,13] The CD56bright cells, generally known to be less mature and with immunoregulatory functions, in fact had the ability to degranulate at levels comparable to CD56dim cells. CD56bright and CD56neg cells produced less IFN-γ than CD56dim cells. Finally, CD56dim and CD56neg cells produced substantially more MIP-1β than their CD56bright counterpart. These trends were also present in HIV-1–infected individuals with some significant differences. First, we found that NK cells from HIV-1–infected individuals exhibited higher spontaneous functional activity compared with cells from HIV-1–uninfected individuals, suggesting a heightened activation state in response to infection. Furthermore, we found that the CD56neg subset of NK cells was functional, in HIV-infected as well as uninfected subjects, with an increased ability to degranulate and produce the CC-chemokine MIP-1β in infected subjects. Previous studies have suggested impaired function of NK cells with focus on the expanded CD56neg subset.[3,5,10,14,20,38] Our data agree with the modest amounts of IFN-γ reported in CD56neg NK cells but would suggest that these cells are not generally dysfunctional. Rather, they are skewed to a different functional profile with the capability to produce large amounts of MIP-1β, although retaining the ability to degranulate. Significant immune pressure on HIV-1 viremia could come from chemokine production and inhibition of viral entry through blocking of CCR5, and eventual mutation and coreceptor switch could be what evades this arm of the immune system.

NK cells receive many activating and inhibitory signals from their environment, and integrated signaling through these

receptors influence what the NK cell will do.[39] One way that HIV-1 may deregulate the immune response is to alter the expression levels of these receptors on the surface of NK cells. Expression of several NCRs, including NKp30, NKp44, and NKp46, has been reported to be significantly decreased in HIV-1 infection.[11] It has also been reported that HIV-1 provokes a switch from the inhibitory C-type lectin NKG2A to the activating form of the receptor, NKG2C, independently of the viral load.[15] Our data concur with these observations of decreased receptor expression. One potential explanation for these changes in the overall NK cell compartment could be loss of CD56bright NK cells, but this possibility is not supported by our data. We observed reductions in NKG2A and NKp30, but these reductions were seen in CD56dim and CD56neg NK cell subsets and not within the CD56bright compartment. Another potential mechanism of NK cell dysregulation might be chronic NK cell activation where increased HLA-DR and CD69 coexpression without the interleukin-2 receptor coincides with NCR depression and decreased cytolytic activity.[40] This might parallel the immune activation seen within the T-cell compartment and lead to eventual immune exhaustion. Further exploration of the role of NK cell activation and exhaustion could prove useful in determining the mechanism of NK cell dysregulation in HIV-1 infection.

Data from simian/HIV (SHN)-infected macaques have suggested that viral coreceptor usage could influence the rate of CD4 decline and expression of the NKp44 ligand on CD4 cells.[41] Upregulation of the NKp44 ligand on CD4 cells as a function of gp41 was also reported to increase CD4 susceptibility to NK lysis.[42] Furthermore, antibodies to a certain sequence of gp41 may abrogate the increased expression of NKp44 ligand on CD4 T cells and decrease their susceptibility to NK cell lysis.[43] In the present study, NKp44 was detected at only very low levels. However, CD56bright NK cells had the highest expression of NKp44 regardless of HIV-1 infection status, and both CD56neg and CD56dim NK cells increased their expression of NKp44 in HIV-1 infection. Interestingly, expression of this receptor in CD56dim NK cells correlated inversely with the CD4 absolute counts in our cohort, indirectly supporting a role for this receptor in disease progression. It is important to note that the role of NKp44 remains unclear in HIV-1 disease as some reports indicate increased, sustained, or decreased expansion of this activating NCR in chronic disease.[11,42,44] More studies are needed to fully understand the potential role of this receptor in HIV-1 disease.

Our observation of elevated IFN-γ production, degranulation, and expression of KIR3 in CD56bright NK cells in patients with low CD4 counts is striking. This subset of NK cells is enriched in the secondary lymphoid organs[45] and may play a role in directing adaptive immune responses.[7] It is therefore interesting that changes in expression of these 3 NK receptors occur when CD4 counts diminish, and one might speculate that alterations in this NK cell subset could be involved in the overall dysregulation of the immune system in progressive disease. The phenotypic and functional changes we have observed in the CD56bright and CD56dim NK cell subsets might be a consequence of the systemic immune activation and loss of T-cell homeostasis associated with HIV-1 progression.[46,47] Alternatively, NK cells might respond directly to high levels of microbial products in plasma. However, it is also possible that these changes are part of the pathogenic process. More studies are needed to fully understand the role of changes in the NK cell compartment in HIV-1 pathogenesis.

A potential bias between the HIV-1–infected and HIV-1–uninfected groups in this study is the HSV-2 infection status. Eighty-two percent of HIV-1–infected subjects were coinfected with HSV-2, whereas only 48% of HIV-1–uninfected subjects carried this virus. However, we observed no statistically significant differences with regard to lymphocyte distribution, NK cell subset distribution, NK cell phenotype, or HIV-1 viral burden in HIV-1–infected subjects with or with out HSV-2 coinfection (data not shown). The same was true for HIV-1–uninfected subjects, although there seemed to be a trend toward increased CD56dim NK cells and decreased CD56neg NK cells in HSV-2–positive subjects (data not shown). In addition, there was no significant difference in surface receptor expression, except for NKp44, which was higher in the HSV-2–infected and HIV-1–uninfected controls ($P = 0.026$).

Together, our data on NK cells in the context of the Ugandan HIV-1 epidemic, which is predominantly composed of clade A and D infections, indicate that infected subjects have an elevated NK cell activity and altered functional and phenotypic NK cell profile. The data furthermore identify NK cell correlates with CD4 cell loss, which suggests that HIV-1 disease progression may be linked with changes in the NK cell compartment. A deeper understanding of how these innate cells work in concert with adaptive immunity could uncover future strategies of HIV-1 therapy and prevention.

ACKNOWLEDGMENTS

The authors would like to thank the Kayunga cohort volunteers for their valuable participation and cooperation during the conduct of this study and the Makerere University Walter Reed Project Staff for their continued dedication toward development of a safe and effective HIV-1 vaccine. In addition, the authors appreciate the strong support that the Kayunga District leadership has shown in working together to fight HIV. Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained are the private views of the author and are not to be construed as official or as reflecting true views of the Department of the Army or the Department of Defense.

REFERENCES

1. Caligiuri MA. Human natural killer cells. *Blood*. 2008;112:461–469.
2. Vivier E, Tomasello E, Baratin M, et al. Functions of natural killer cells. *Nat Immunol*. 2008;9:503–510.
3. Alter G, Altfeld M. NK cell function in HIV-1 infection. *Curr Mol Med*. 2006;6:621–629.
4. Biassoni R, Cantoni C, Pende D, et al. Human natural killer cell receptors and co-receptors. *Immunol Rev.* 2001;181:203–214.
5. Fauci AS, Mavilio D, Kottilil S. NK cells in HIV infection: paradigm for protection or targets for ambush. *Nat Rev Immunol*. 2005;5:835–843.
6. Kärre K. Natural killer cell recognition of missing self. *Nat Immunol*. 2008;9:477–480.
7. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood*. 2001;97:3146–3151.

8. Alter G, Teigen N, Ahern R, et al. Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. *J Infect Dis*. 2007; 195:1452–1460.
9. Hu PF, Hultin LE, Hultin P, et al. Natural killer cell immunodeficiency in HIV disease is manifest by profoundly decreased numbers of CD16+CD56+ cells and expansion of a population of CD16dimCD56- cells with low lytic activity. *J Acquir Immune Defic Syndr Hum Retrovirol*. 1995;10:331–340.
10. Azzoni L, Papasavvas E, Chehimi J, et al. Sustained impairment of IFN-gamma secretion in suppressed HIV-infected patients despite mature NK cell recovery: evidence for a defective reconstitution of innate immunity. *J Immunol*. 2002;168:5764–5770.
11. De Maria A, Fogli M, Costa P, et al. The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). *Eur J Immunol*. 2003;33:2410–2418.
12. Alter G, Teigen N, Davis BT, et al. Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection. *Blood*. 2005;106:3366–3369.
13. Gonzalez VD, Falconer K, Michaëlsson J, et al. Expansion of CD56- NK cells in chronic HCV/HIV-1 co-infection: reversion by antiviral treatment with pegylated IFNalpha and ribavirin. *Clin Immunol*. 2008;128:46–56.
14. Mavilio D, Lombardo G, Benjamin J, et al. Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proc Natl Acad Sci U S A*. 2005;102:2886–2891.
15. Mela CM, Burton CT, Imami N, et al. Switch from inhibitory to activating NKG2 receptor expression in HIV-1 infection: lack of reversion with highly active antiretroviral therapy. *AIDS*. 2005;19:1761–1769.
16. Bandyopadhyay S, Ziegner U, Campbell DE, et al. Natural killer cell-mediated lysis of T cell lines chronically infected with HIV-1. *Clin Exp Immunol*. 1990;79:430–435.
17. Bonaparte MI, Barker E. Killing of human immunodeficiency virus-infected primary T-cell blasts by autologous natural killer cells is dependent on the ability of the virus to alter the expression of major histocompatibility complex class I molecules. *Blood*. 2004;104:2087–2094.
18. Fehniger TA, Herbein G, Yu H, et al. Natural killer cells from HIV-1+ patients produce C-C chemokines and inhibit HIV-1 infection. *J Immunol*. 1998;161:6433–6438.
19. Oliva A, Kinter AL, Vaccarezza M, et al. Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. *J Clin Invest*. 1998;102:223–231.
20. Kottilil S, Chun TW, Moir S, et al. Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function. *J Infect Dis*. 2003;187:1038–1045.
21. Martin MP, Gao X, Lee JH, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet*. 2002;31: 429–434.
22. Long BR, Ndhlovu LC, Oksenberg JR, et al. Conferral of enhanced natural killer cell function by KIR3DS1 in early human immunodeficiency virus type 1 infection. *J Virol*. 2008;82:4785–4792.
23. Alter G, Martin MP, Teigen N, et al. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med*. 2007;204:3027–3036.
24. Ballan WM, Vu BN, Long BR, et al. 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. *J Immunol*. 2007;179:3362–3370.
25. Jennes W, Verheyden S, Demanet C, et al. Cutting edge: resistance to HIV-1 infection among African female sex workers is associated with inhibitory KIR in the absence of their HLA ligands. *J Immunol*. 2006;177: 6588–6592.
26. Wakabi W. New strategies sought in Uganda as HIV infections rise. *Lancet Infect Dis*. 2008;8:285.
27. Hladik W, Musinguzi J, Kirungi W, et al. The estimated burden of HIV/AIDS in Uganda, 2005-2010. *AIDS*. 2008;22:503–510.
28. Harris M, Serwada D, Sewankambo NK, et al. Among 46 near full-length HIV type 1 genome sequences from Rakai District, Uganda, subtype D and AD recombinants predominate. *AIDS Res Hum Retroviruses*. 2002; 18:1281–1290.
29. Peeters M, Toure-Kane C, Nkengasong JN. Genetic diversity of HIV in Africa: impact on diagnosis, treatment, vaccine development and trials. *AIDS*. 2003;17:2547–2560.
30. Kiwanuka N, Laeyendecker O, Robb M, et al. Effect of human immunodeficiency virus type 1 (HIV-1) subtype on disease progression in persons from Rakai, Uganda, with incident HIV-1 infection. *J Infect Dis*. 2008;197:707–713.
31. Baker CA, McEvers K, Byaruhanga R, et al. HIV subtypes induce distinct profiles of HIV-specific CD8(+) T cell responses. *AIDS Res Hum Retroviruses*. 2008;24:283–287.
32. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods*. 2004;294: 15–22.
33. Gonzalez VD, Björkström NK, Malmberg KJ, et al. Application of nine-color flow cytometry for detailed studies of the phenotypic complexity and functional heterogeneity of human lymphocyte subsets. *J Immunol Methods*. 2008;330:64–74.
34. Eller LA, Eller MA, Ouma BJ, et al. Large-scale human immunodeficiency virus rapid test evaluation in a low-prevalence Ugandan blood bank population. *J Clin Microbiol*. 2007;45:3281–3285.
35. Laeyendecker O, Henson C, Gray RH, et al. Performance of a commercial, type-specific enzyme-linked immunosorbent assay for detection of herpes simplex virus type 2-specific antibodies in Ugandans. *J Clin Microbiol*. 2004;42:1794–1796.
36. Arroyo MA, Sateren WB, Serwadda D, et al. Higher HIV-1 incidence and genetic complexity along main roads in Rakai District, Uganda. *J Acquir Immune Defic Syndr*. 2006;43:440–445.
37. Hoelscher M, Dowling WE, Sanders-Buell E, et al. Detection of HIV-1 subtypes, recombinants, and dual infections in East Africa by a multi-region hybridization assay. *AIDS*. 2002;16:2055–2064.
38. Mavilio D, Benjamin J, Daucher M, et al. Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates. *Proc Natl Acad Sci U S A*. 2003; 100:15011–15016.
39. Bryceson YT, Long EO. Line of attack: NK cell specificity and integration of signals. *Curr Opin Immunol*. 2008;20:344–352.
40. Fogli M, Costa P, Murdaca G, et al. Significant NK cell activation associated with decreased cytolytic function in peripheral blood of HIV-1-infected patients. *Eur J Immunol*. 2004;34:2313–2321.
41. Vieillard V, Habib RE, Brochard P, et al. CCR5 or CXCR4 use influences the relationship between CD4 cell depletion, NKp44L expression and NK cytotoxicity in SHIV-infected macaques. *AIDS*. 2008;22:185–192.
42. Vieillard V, Strominger JL, Debré P. NK cytotoxicity against CD4+ T cells during HIV-1 infection: a gp41 peptide induces the expression of an NKp44 ligand. *Proc Natl Acad Sci U S A*. 2005;102:10981–10986.
43. Vieillard V, Costagliola D, Simon A, et al; Group FAàLTAS. Specific adaptive humoral response against a gp41 motif inhibits CD4 T-cell sensitivity to NK lysis during HIV-1 infection. *AIDS*. 2006;20: 1795–1804.
44. Fu GF, Hao S, Zhao JL, et al. Changes in NK cell counts and receptor expressions and emergence of CD3(dim)/CD56+ cells in HIV-1 infected patients in China. *Viral Immunol*. 2009;22:105–116.
45. Strowig T, Brilot F, Münz C. Noncytotoxic functions of NK cells: direct pathogen restriction and assistance to adaptive immunity. *J Immunol*. 2008;180:7785–7791.
46. Hunt PW, Brenchley J, Sinclair E, et al. Relationship between T cell activation and CD4+ T cell count in HIV-seropositive individuals with undetectable plasma HIV RNA levels in the absence of therapy. *J Infect Dis*. 2008;197:126–133.
47. Jiang W, Lederman MM, Hunt P, et al. Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection. *J Infect Dis*. 2009;199:1177–1185.