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An NK Cell Population Lacking FcRγ Is Expanded in Chronically Infected HIV Patients

Jingling Zhou,* Fathiah S. Amran,† Marit Kramska,‡ Tom A. Angelovich,*§ Julian Elliott,¶ Anna C. Hearps,*¶ Patricia Price,†,1 and Anthony Jaworowski*,*†,‖

We previously demonstrated that NK cells from HIV-infected individuals have elevated expression of activation markers, spontaneously degranulate ex vivo, and decrease expression of a signal-transducing protein for NK-activating receptors, FcRγ. Importantly, these changes were maintained in vireologically suppressed (VS) individuals receiving combination antiretroviral therapy (cART). In this study, we show that loss of FcRγ is caused by the expansion of a novel subset of FcRγ−CD56dim NK cells with an altered activation receptor repertoire and biological properties. In a cross-sectional study, FcRγ− NK cells as a proportion of total CD56dim NK cells increased in cART-naive viremic HIV-infected individuals (median [interquartile range] = 25.9 [12.6–56.1] compared with 3.80 [1.15–11.5] for HIV− controls, p < 0.0001) and in VS HIV-infected individuals (22.7 [13.1–56.2] compared with 3.80 [1.15–11.5], p = 0.0004), with no difference between cART-naive and VS patients (p = 0.93). FcRγ− NK cells expressed no NKp30 or NKp46. They showed greater Ab-dependent cellular cytotoxicity activity against rituximab-opsonized Raji cells and in a whole-blood assay measuring NK responses to overlapping HIV peptides, despite having reduced CD16 expression compared with conventional NK cells. Their prevalence correlated with CMV Ab titers in HIV+ individuals, and with the inflammatory marker CXCL10 in both groups. The expansion of a subset of NK cells that lacks NKp30 and NKp46 to ~90% of CD56dim NK cells in some VS HIV+ individuals may influence NK-mediated immunosurveillance in patients receiving cART. The Journal of Immunology, 2015, 194: 000–000.

C ombination antiretroviral therapy (cART) reduces HIV viremia in peripheral blood to levels undetectable by standard clinical assays (<20 copies HIV RNA/ml) and reduces the incidence of AIDS and associated mortality. However cART does not eliminate HIV infection, and treated individuals experience elevated rates of non-AIDS comorbidities including non-AIDS cancers such as Hodgkin’s lymphoma and solid tumors (1), demonstrating that cART does not fully restore functional immunity. Understanding why immune defects persist in HIV+ individuals receiving cART will facilitate adjunctive therapies to improve long-term health outcomes in HIV-infected individuals.

Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; ART, antiretroviral therapy; cART, combination antiretroviral therapy; CMVgB, CMV glycoprotein B; CMVIE-1, CMV immediate early Ag-1; gB, glycoprotein B; HFF, human foreskin fibroblast; hsCRP, high-sensitivity C-reactive protein; IE-1, immediate early Ag-1; IQR, interquartile range; MFI, mean fluorescence intensity; VS, virologically suppressed.

We and others have shown activation of the innate immune system in patients who achieve virologic suppression on cART, exemplified by elevation of phenotypic and plasma markers of activation related to innate immune cells, notably monocytes/macrophages (2–8). “Elite controllers” (HIV+ individuals who control HIV viremia without ART) also display innate immune activation (9), suggesting it may be independent of HIV viremia and cART. Instead, the causes may include residual HIV replication in tissues (10, 11), reactivation of latent viruses such as CMV (12, 13), and/or the presence of circulating bacterial products due to loss of gut integrity (4).

NK cells are innate lymphoid cells (14) that kill transformed and virus-infected cells via perforin/granzyme and Fas/Fasl-dependent mechanisms. Human NK cells are divided into subsets defined by expression of CD56 (NCAM-1), an adhesion molecule that participates in homotypic interactions, and CD16 (FcγRIIIa), an activating receptor for IgG-promoting Ab-dependent cellular cytotoxicity (ADCC). CD56dimCD16+ NK cells are less mature and constitute a minor subset in circulation but more prominent in secondary lymphoid tissues (15). They have relatively poor cytotoxic activity but are robust producers of inflammatory cytokines and chemokines in response to cytokine stimulation. CD56dimCD16+ NK cells are more mature, secrete inflammatory cytokines such as IFN-γ in response to cytotoxic targets, and are more abundant in circulation. They exhibit cytotoxic and CD16-dependent ADCC activity. In chronic viral infections, such as HIV, a senescent population of CD56− NK cells accumulates in circulation (16, 17). These cells proliferate poorly, have limited cytotoxic ability and proinflammatory cytokine secretion, but retain the ability to secrete chemokines such as CCL4 (18, 19).

NK activation receptors signal via small adaptor proteins containing ITAM motifs, namely TCRζ, FcRγ, and DAP12 (20). ITAM motifs on these proteins are phosphorylated following engagement of the activation receptors and act as docking sites.
for spleen tyrosine kinase and ZAP70, which in turn activate downstream signaling pathways such as those required for cytokine production and degranulation (21). The ITAM-containing signaling proteins also act as chaperones and are essential for surface expression of their cognate activating receptors.

The role of NK cells as antiviral effectors has prompted studies into how their numbers, subset distribution, phenotype, and activity are altered in HIV disease. NK cell numbers increase during acute HIV infection largely due to expansion of the CD56bright subset (22), whereas the numbers of CD56dim NK cells appear to be relatively unaffected (16). Viremic HIV disease is characterized by expansion of CD56dim NK cells, which disappear in virologically suppressed (VS) individuals receiving cART (23). HIV infection is also characterized by the loss of NKG2A+ NK cells and increases in NKG2C+ NK cells (24). Both NKG2A and NKG2C form heterodimers with CD94 and recognize HLA-E; however, the NKG2A heterodimer is an inhibitory receptor whereas the latter promotes activation. Although changes in the proportion of NKG2A and NKG2C+ NK cells are independent of viral load (24), they may be driven by CMV and were reversed following virologic suppression on cART (25). NK natural cytotoxic activity against the heterologous target cell K562 is decreased in viremically infected HIV but was restored in one study following virologic suppression within 2 wk of initiating cART (26).

Whereas HIV viremia is usually well managed by cART, patients retain innate immune activation and inflammation that may influence NK activity and function. We have shown that their NK cells express elevated phenotypic markers of cell activation, spontaneously degranulate ex vivo, and have altered signal transduction, which persist following virologic suppression (27, 28). In particular, HIV infection was associated with a significant decrease in expression of FcRγ in NK cells, which varies in CD56dim NK subset that persists in VS individuals and represents up to 90% of the total CD56dim population. We compare the phenotype of FcRγ+ and conventional NK cells and investigate the role of CMV and inflammation in their expansion in HIV+ individuals.

Materials and Methods

Study group

HIV-seropositive individuals were recruited and blood was taken with informed consent by research staff in the Department of Infectious Diseases, Alfred Hospital (Melbourne, VIC, Australia). Ethical approval was obtained from the Alfred Hospital Research and Ethics Committee. Study subjects included 10 persons not currently receiving cART (mean [SD] viral load = 72,351 [47,565], range = 17,500–153,200 copies/ml), 33 persons receiving cART at the time of sampling (1 with a viral load of 150 copies/ml and the remainder with a viral load of ≤50 copies/ml). As controls, 23 HIV-seronegative control subjects were recruited from the community and blood was taken by an experienced phlebotomist.

Antibodies

CD3 PerCP-Cy5.5 (clone UCHT1), CD16 PE-Cy7 (clone 3G8), CD335 PE (clone p48-3.1), CD107a allophycocyanin-H7 (clone H4A3), TNF PE (clone Mab11), and IFN-γ Alexa Fluor 488 (clone B27) were from BD Biosciences; CD3 Alexa Fluor 700 (clone UCHT1), CD3 BV510 (clone OKT3), CD56 Alexa Fluor 700 (clone HCD 56), CD314 PE (clone 1D11), CD57 Pacific Blue (clone HCD57), CD335 PerCP-Cy5.5 (clone 9E2), and CD337 Alexa Fluor 647 (clone P30-15) were from BioLegend; CD56 allophycocyanin (clone N90/1HLD6A), CD159a allophycocyanin (clone Z199), and TCRζ PE (clone 2H2D9) were from Beckman Coulter; CD159c PerCP (clone 134591) was from R&D Systems; and FITC-conjugated rabbit polyclonal Ab to FcRγ (Milli-Mark, anti-FcRC1 Ab, γ subunit–FITC, catalog no. FCABS400F) was from Millipore.

Cells and plasma were prepared from whole blood collected into EDTA anticoagulant tubes and PBMC were prepared by Ficoll density gradient centrifugation. Cells (1 × 10⁶) were stained with appropriate cell surface markers (CD3, CD56, and CD16) for 30 min on ice, permeabilized (Perm/ Wash buffer 1, BD Biosciences), and stained for FcRγ or TCRζ for 30 min on ice, then washed (Perm/Wash buffer 1, BD Biosciences) and fixed. Phenotyping of NK cell subsets was performed by staining with desired cell surface markers or relevant isotype controls, plus the lineage markers detailed above, before intracellular staining.

CD107a mobilization and intracellular cytokine production

CD107a mobilization in response to CD16 cross-linking was measured as described previously (21). Briefly, CD16 was cross-linked by the addition of 50 μg/ml anti-CD16 (clone 3G8, donated by Mark Hogarth, Burnet Institute, Melbourne, VIC, Australia) to 100 μl whole blood and incubation on ice for 10 min. Cells were washed once with ice-cold PBS, incubated with 8 μl goat anti-mouse F(ab')² fragment (55487, ICN Pharmaceuticals/Cappel, Costa Mesa, CA) for a further 5 min, and then transferred to a 37˚C water bath for the remainder of the incubation. To measure TNF production, 5 × 10⁵ PBMC were incubated in 1 ml RF10 (RPMI 1640 containing 10% newborn calf serum [HyClone, Cosmic Calf serum], 100 U/ml penicillin/streptomycin [Life Technologies], and 2 mM l-glutamine) with an equal number of Raji cells either unopsonized or opsonized with 10 ng/ml rituximab. One hundred microliter RF10 containing anti–CD107a-allophycocyanin-Cy7, 20 μg/ml brefeldin A, and 10 μm monensin was added, and the cells were incubated for 4 h at 37˚C, stained with anti–CD3-PerCP-Cy5.5, anti–CD16-PE-Cy7, and anti–CD56-allophycocyanin (30 min permeabilized, Perm/Wash buffer 1, BD Biosciences). After 10 min on ice, cells were blocked for a further 10 min using 10 μl rabbit serum plus 10 μl mouse serum and then stained with anti–TNF-PE and rabbit anti-FcγR-FTC for 30 min, washed, and fixed. ADCC activity against peptide-pulsed target cells was also measured in a whole-blood assay (29). Briefly, 150 μl fresh whole blood was pulsed with 1 μg/ml of an envelope peptide pool (15 aa each with 11 aa subtype: B, strain MN, National Institutes of Health AIDS Reagent Program) and 25% serum from a single HIV+ donor showing high ADCC activity, in the presence of anti–CD107a-allophycocyanin-Cy7 (BD Biosciences), 10 μg/ml brefeldin A (Sigma-Aldrich), plus 5 μM monensin (GolgiStop, BD Biosciences). After 5 h, cells were stained with anti–CD3-PerCP and anti–CD56-PE-Cy7 and then permeabilized (FACS Perm II, BD Biosciences) and stained for FcγRγ.

Measurement of plasma inflammatory markers

Plasma concentrations of soluble CD163 (Macro 163, Trillium Diagnostics), CXCL10 (CXCL10/IP-10 Quantikine ELISA, R&D Systems), and IFN-α (human IFN-α [pan specific] ELISApro, Mabtech) were measured using commercial ELISA kits according to manufacturers’ instructions. High-sensitivity C-reactive protein (hsCRP) was measured using the Abbott Architect ci16200 (Abbott Laboratories) by the Alfred Pathology Service, Alfred Hospital (Melbourne, VIC, Australia).

CMV DNA, Ab, and total Ig ELISAs

Selected plasma samples were screened for CMV DNA using the COBAS AmpliPrep/COBAS TaqMan CMV test by the Alfred Pathology Service, Alfred Hospital (Melbourne, VIC, Australia). IgG reactive with CMV was quantified using CMV lysate, CMV glycoprotein B (CMVgB), and CMV immediate early Ag-1 (CMVIE-1) Ags. CMV lysate was prepared by sonication of human foreskin fibroblasts (HFF) infected with AD169. Uninfected HFF were prepared and extracts were analyzed in parallel. Replicate ELISA plates were coated with CMVgB (produced in hamster ovary cells, Chiron, 2800-800; 50 ng/ml) and CMVIE-1 (produced in Escherichia coli, Miltenyi Biotec, 130-092-137; 2500 ng/ml). Plasma samples were diluted from 1:200. Binding was detected using goat anti-human IgG–conjugated horseradish peroxidase, followed by tetramethylbenzidine substrate (Accustain, Pierce). Reactions were stopped with 1 M H₂SO₄ and measured at 450 nm. Four-parameter logistic curves were generated from titrations of a plasma sample assigned a value of 100 arbitrary units IgG reactive with each Ag using SoftMax Pro version 5.4 software. The standard was run on each plate and unit values were derived for all samples. The standard curve of the positive plate was used to generate units of Ab on plates coated with uninfected HFF. These were subjected to those gates used for CMV-positive samples. Cell count was quantified using plates coated with polyvalent goat anti-human IgG (2.5 mg/ml; Invitrogen) diluted 1:500 in bicarbonate buffer and blocked with 5% BSA in PBS for 60 min. Plasma samples were diluted from 1:100,000.
in 2% BSA/PBS and applied for 120 min. Binding was detected using goat anti-human IgG-conjugated HRP (Sigma-Aldrich) followed by tetramethylbenzidine substrate as described above.

Statistical analyses
Comparisons between HIV− and HIV+ groups were made using a non-parametric Mann–Whitney U test. Differences in properties of FcRγ− and conventional NK cells within HIV− patients and controls were assessed using a paired Wilcoxon signed rank test. To measure the association between inflammatory biomarkers or CMV Abs and the proportion of FcRγ− NK cells, data were log transformed (where required) to achieve a normal distribution (D’Agostino–Pearson omnibus normality test), and Pearson correlation coefficients were determined from the transformed data. Lines of best fit were computed separately using data from HIV− and HIV+ individuals and their slopes were compared. All analyses were performed using GraphPad Prism, version 6.0.

Results
An FcRγ CD56<sub>dim</sub> NK cell population is present in HIV+ individuals
We have previously reported that FcRγ expression in NK cells from HIV+ individuals is decreased irrespective of CD4 T cell count or HIV viral load (21, 27, 28). To identify the reason for this decrease, we examined expression of FcRγ and the functionally related signaling molecule TCRζ in NK cell subsets. In healthy control subjects, CD56<sub>dim</sub> and CD56<sub>bright</sub> NK cells expressed both FcRγ and TCRζ (Fig. 1A, red and gray contour plots, respectively). The FcRγ CD56<sub>dim</sub> cells were further characterized as NK cells because they also lacked expression of monocyte (CD14), T cell (CD3), and B cell (CD19) markers (Supplemental Fig. 1). The level of FcRγ expression coincided with negative staining using control FITC-conjugated rabbit IgG (Supplemental Fig. 1) and with the level of staining in CD3+ T lymphocytes, most of which do not express FcRγ (not shown). The expression of FcRγ and TCRζ was correlated in both NK cell subsets, but it was lower in the CD56<sub>bright</sub> NK cells. We also examined FcRγ expression in monocytes and showed that expression was elevated in both subsets defined by CD16 (nonclassical and intermediate) compared with classical monocytes, whereas all three subsets had comparable expression of TCRζ (Supplemental Fig. 2). In HIV+ subjects, CD56<sub>dim</sub> NK cells showed two populations that differed in either expressing or not expressing FcRγ while having equivalent levels of TCRζ expression (Fig. 1B, red contour plot). In contrast, FcRγ expression was normal in the CD56<sub>bright</sub> population (Fig. 1B, gray contour plot). These data show that HIV infection was associated with the selective loss of FcRγ expression in CD56<sub>dim</sub> NK cells. We next examined the prevalence of FcRγ CD56<sub>dim</sub> NK cells (gated as shown in Fig. 1C and hereafter called FcRγ− NK cells) in a cross-sectional study comprising 23 HIV−, 10 viremic HIV+, and 33 VS HIV+ individuals (Table I). The FcRγ− population was greatly expanded in viremic HIV+ individuals compared with HIV-uninfected individuals (median interquartile range, IQR) = 25.9 [12.6–56.1] compared with 3.80 [1.15–11.5], p = 0.0009) and remained unchanged following virologic suppression (median IQR = 22.7 [13.1–56.2], p < 0.0001 compared with HIV-uninfected and p = 0.927 compared with HIV+ viremic) (Fig. 1D). We examined FcRγ expression in all three monocyte subsets in a subset of the study participants and found no differences (Supplemental Fig. 3A). We also determined that TCRζ expression was unchanged by HIV infection in monocytes (Supplemental Fig. 3B) and in CD56<sub>bright</sub> and CD56<sub>dim</sub> NK cells (Supplemental Fig. 3C). Considered together, these data support the conclusion that HIV infection selectively affects FcRγ expression in NK cells.

Prevalence of FcRγ− (CD56<sub>dim</sub>) NK cells is associated with Ab levels to CMV Ags and plasma markers of inflammation
The proportion of FcRγ− NK cells in VS HIV+ individuals did not correlate with their duration of ART (Pearson’s r = −0.099 p = 0.589). Neither was there any correlation with current CD4 (r = −0.134, p = 0.415) or nadir CD4 (r = −0.017, p = 0.916) counts. FcRγ−deficient NK cells are present in low abundance in otherwise healthy blood donors who are seropositive for CMV (30). CMV seropositivity is >90% among HIV patients and may be used as a marker of their historical burden of CMV. In the present study, we measured Ab responses by ELISA using a lysate of CMV-infected fibroblasts (HFF), CMVgB, and the latency-associated CMVIE-1. HIV+ individuals had elevated levels of Ab to CMV lysate and gB, but not to IE-1 (Fig. 2A). Because CMV reactivation can increase with age, and the median age of VS HIV+ individuals in our cohort was older than viremic HIV+ or uninfected individuals (Table I), we analyzed CMV Abs in a subset of participants aged ≤45 y to determine whether the above-mentioned effect was influenced by age. This analysis confirmed increased levels of Abs to both CMV HFF lysate (p = 0.003) and gB (p < 0.0001) in VS HIV+ individuals as compared with controls of a similar age (Table II). Although there was a trend toward higher levels of total IgG in plasma from HIV+ individuals (0.077 and 0.059 for viremic and VS, respectively), these differences were not significant, suggesting that the increase in Abs to CMV is not solely attributed to hypergammaglobulinemia. The percentage of FcRγ− NK cells correlated strongly with the levels of Ab to HFF (Pearson’s r = 0.676, p < 0.0001) and gB (r = 0.597, p < 0.0001) and moderately with IE-1 (r = 0.343, p = 0.0091) (Fig. 2B). However, when subjects were stratified according to their HIV status, there was a significant correlation between total CMV lystate Ab and FcRγ− NK cells in controls (r = 0.759, p = 0.0002) but not in HIV+ subjects (r = 0.024, p = 0.90). We also performed linear regression analysis on the relationship between FcRγ− NK cells and HFF Ab levels and adjusted this model for age. This analysis confirmed significantly higher levels of CMV Abs in viremic and VS HIV+ individuals (p = 0.002 and 0.015, respectively), independent of age.

We next measured plasma markers of inflammation (CXCL10, soluble CD163, hsCRP, soluble TNFR), and IFN-α. In this small cross-sectional group soluble CD163 and CXCL10 were significantly elevated in viremic HIV+ individuals and remained elevated in VS patients (2), whereas hsCRP, soluble TNFR, and IFN-α were not significantly different (Fig. 3A–E). Only CXCL10 correlated with the prevalence of FcRγ− NK cells (Pearson’s r = 0.550, p < 0.0001; Fig. 3F). Similarly, there was a strong correlation between CXCL10 and FcRγ− NK cells in HIV+ individuals (r = 0.579, p = 0.015) but the correlation in HIV+ individuals did not reach significance (r = 0.313, p = 0.060). It is of interest that, although elevated in HIV+ individuals, there was no relationship between FcRγ− NK cells and soluble CD163.

FcRγ− NK cells in HIV+ individuals exhibit enhanced ADCC activity
FcRγ expression is associated with CD16-dependent ADCC activity (21); therefore, we compared ADCC activity in FcRγ− and conventional NK cells. PBMC from VS HIV+ individuals were exposed to rituximab-opsonized or nonopsonized Raji cells and degranulation was assessed using cell surface CD107a. FcRγ− NK cells showed greater degranulation in response to both nonopsonized and rituximab-opsonized Raji cells (Fig. 4A). To confirm these findings, NK degranulation and IFN-γ production in response to a HIV peptide pool were measured in whole blood from VS HIV+ individuals (29). The assay was modified to gate on FcRγ− and conventional NK cells. FcRγ− NK cells degranulated...
and produced more IFN-γ in response to overlapping HIV peptide pools than did conventional NK cells (Fig. 4B). Overall, FcγR NK cells present in HIV+ individuals have higher ADCC activity than do conventional NK cells.

**CD38 expression is lower and spontaneous NK cell degranulation is similar in FcγR⁺ NK cells compared with conventional NK cells**

We have shown that NK cells from VS HIV+ individuals have higher expression of the lymphocyte activation marker CD38 and spontaneously degranulate ex vivo (28). We compared these parameters in both FcγR⁺ and conventional NK cells to determine whether our previous observations could be explained by the high prevalence of FcγR⁺ NK cells in HIV patients. Using an ex vivo whole-blood assay we confirmed NK cells from VS HIV patients degranulate at a higher rate than do those from HIV− control subjects, but the rate of spontaneous degranulation did not differ between FcγR⁺ and conventional NK cells (Fig. 4C). FcγR⁺ NK cells expressed low levels of HLA-DR, similar to conventional NK cells, but expressed less CD38 (Fig. 4D). These data suggest

### Table I. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>HIV Uninfected</th>
<th>Viremic</th>
<th>VS</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>23</td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td>Age (y)</td>
<td>33.5 (30.8–49.8)</td>
<td>33.0 (26.8–38.0)</td>
<td>46.0 (34.5–54.0)</td>
</tr>
<tr>
<td>CD4 T cells/μl</td>
<td>N.A.</td>
<td>400 (309–457)</td>
<td>493 (378–802)</td>
</tr>
<tr>
<td>HIV RNA copies/ml plasma</td>
<td>N.A.</td>
<td>52,100 (37,378–109,510)</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Nadir CD4</td>
<td>N.A.</td>
<td>309 (281–374)</td>
<td>244 (191–331)</td>
</tr>
<tr>
<td>Time on cART (y)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>6.65 (2.34–16.0)</td>
</tr>
</tbody>
</table>

All study participants were male. VS HIV+ patients were older than viremic HIV+ patients \( (p = 0.009) \). Values provided are median (IQR). N.A., not applicable.
that increased NK cell activation in VS HIV+ individuals, measured phenotypically or via degranulation ex vivo, is not due to an increased proportion of FcRγ2 NK cells in these individuals, as it is apparent in both this subset and in conventional NK cells.

Expression of NKG2 receptors on FcRγ2 and conventional NK cells

Because chronic HIV infection alters the NKG2A/NKG2C ratio on NK cells in patients who are CMV seropositive (25), we assessed NKG2A and NKG2C on FcRγ2 and conventional NK cells. FcRγ2 NK cells had lower NKG2A expression in most individuals (Fig. 5A), whereas NKG2C expression was equivalent in both populations (Fig. 5B).

Phenotypic characterization of FcRγ− NK cells

Both FcRγ and TCRz have been shown in transfected cell lines and using knockout mice to act as molecular chaperones for the receptors for which they transduce signals (31–35), but their role as chaperones in primary human NK cells is less clear. We assessed NK-activating receptors, as well as maturation and senescence markers, on FcRγ− and conventional NK cells. FcRγ− NK cells did not express NKp30 or NKp46 (Fig. 5C), consistent with a role for FcRγ as a chaperone for these NK-activating receptors.

FcRγ− NK cells expressed CD16, but with lower mean fluorescence intensity (MFI) (Fig. 5D), whereas CD56 expression was comparable (Fig. 5E), which does not fit with the normal differentiation of CD56bright to CD56dim cells in which CD16 expression increases and CD56 expression decreases. CD62L, expressed on a population of less mature, polyfunctional CD56dim NK cells (36), was expressed on a minority of NK cells, but CD62LFcRγ− NK cells had a lower MFI than did CD62L+ conventional NK cells (Fig. 5F). More FcRγ− NK cells expressed the maturation marker CD57 (37) but fewer expressed the TNFR family member CD27 (Fig. 5G and 5H, respectively). It was difficult to assess the phenotype of FcRγ− NK cells in HIV2 control subjects, as the populations were small, but no differences were identified (Supplemental Fig. 4).

Discussion

In this study, we associate HIV infection with expansion of an FcRγ− NK cell population that lacks expression of the activating receptors NKp30 and NKp46. FcRγ− NK cells represent up to 90% of the total CD56dim NK cell pool in viremic HIV+ individuals and patients on cART for 6.5 y. Expansion of the FcRγ− NK cell population did not correlate with CD4 counts, nadir CD4 counts, plasma HIV RNA, or time on cART, as observed when we measured FcRγ expression in the total NK cell population (28). Hence, our data suggest that FcRγ− NK cell expansion is not due to HIV infection per se, nor is it a direct consequence of the immunological damage caused by HIV before cART. Our finding

Table II. CMV Ab titers in participants aged ≤45 y

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Viremic HIV+</th>
<th>VS HIV+</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Age (y), median (range)</td>
<td>31.0 (27–45)</td>
<td>30.0 (24–38)</td>
<td>35.5 (29–46)</td>
</tr>
<tr>
<td>CMV HFF Ab titer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>844 (50–3,455)</td>
<td>5,411 (4,115–41,804)</td>
<td>10,111 (2,006–23,653)</td>
</tr>
<tr>
<td>p Value</td>
<td>—</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>CMVgB Ab titer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>46 (28–379)</td>
<td>4,345 (2,464–5,036)</td>
<td>2,835 (1,454–4,824)</td>
</tr>
<tr>
<td>p Value</td>
<td>—</td>
<td>0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CMVIE-1 Ab titer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>880 (447–1,303)</td>
<td>1,820 (973–3,279)</td>
<td>842 (396–3,865)</td>
</tr>
<tr>
<td>p Value</td>
<td>—</td>
<td>0.019</td>
<td>NS</td>
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</table>

The p values show comparisons versus controls via a Mann–Whitney U test.
that FcRγ expression is decreased at the level of mRNA and protein suggests that the FcRγ gene expression is reduced, rather than epitopes recognized by the FcRγ Ab used for intracellular flow cytometry being masked.

Similar to an FcRγ2 NK cell subset described in CMV-seropositive blood donors (30), the population we describe in the present study has enhanced ADCC activity relative to conventional NK cells despite lower expression of CD16, so CD16 expression is not invariably rate determining for ADCC activity. Because CD16 can signal via both FcRγ and TCRζ, ADCC signaling may be transduced by TCRζ in FcRγ-deficient cells and signaling via FcRγ and/or TCRζ may be rate determining. CD16 expression on NK cells is reduced in HIV+ individuals via ADAM17-dependent shedding (38, 39) but it is not clear to what extent ADAM17 reduces CD16 expression in FcRγ2 NK cells and how much loss of CD16 is due to loss of FcRγ chaperone function. NK cells from ~40% of individuals express the activating Fcγ receptor, CD32c, which can mediate ADCC via its own cytoplasmic ITAM motif (40). It is possible that differential expression of this receptor between NK subsets might account for the higher ADCC activity of the FcRγ− population observed in some HIV patients, but the has not been examined in the present study. The expansion of a population of NK cells with increased ADCC activity in HIV+ individuals has relevance to vaccine strategies aimed at generating ADCC-promoting Ab responses (41–43).

Other NK cell abnormalities have been associated with CMV coinfection in HIV+ individuals. In particular, NK cells displayed skewed expression of NKG2A and NKG2C that normalized after 24 mo cART (25). Given the presence of FcRγ− NK cells in CMV-seropositive individuals (30), we considered whether this is the same population. Although FcRγ− NK cells generally expressed low levels of NKG2A, this is not true for all individuals. Similarly, NKG2C expression was similar to that on conventional NK cells within any individual. Thus, although there is overlap with the NK cell subset expanded in CMV HIV-coinfected individuals, these are not congruent subsets. FcRγ− NK cells in HIV+ individuals share many characteristics with mature CD56dim NK cells that lose NKG2A, have lower proliferative capacity, and acquire CD57 and KIR expression (37). Unlike these cells, FcRγ− NK cells do not lose CD62L expression, a marker that defines polyfunctional and less mature CD56dim NK cells (36).
presence of these cells in VS HIV+ individuals, their high ADCC activity, and their high CD56 expression are not consistent with their being precursors to dysfunctional CD56dim NK cells that accumulate in viremic HIV individuals (17). Whether they represent a terminally differentiated CD56dim NK cell population that normally does not accumulate in the periphery of healthy individuals remains to be determined.

Although FcRγ− NK cells have been associated with CMV seropositivity in HIV− individuals (30), all of the HIV− individuals used in this study were seropositive for CMV, so this alone cannot explain the variation in prevalence of FcRγ− NK cells in the setting of HIV infection. Analysis of the plasma from five VS HIV+ individuals who had no or very low FcRγ− NK cells and five individuals with a very high proportion of FcRγ− NK cells showed no detectable CMV viral load using standard clinical assays (data not shown). We next examined the relationship between FcRγ− NK cells and CMV infection in our HIV+ patients and control subjects by correlating their proportion with levels of CXCL10 in both HIV− and HIV+ individuals. Rather, the correlation with levels of CXCL10 in both HIV− and HIV+ individuals implicates chronic inflammation. In support of this, we have detected high proportions of FcRγ− NK cells in heart transplant patients who receive prophylactic valganciclovir to inhibit CMV reactivation (A.C. Hearps, unpublished), suggesting that the subset is not expanded as a result of current CMV reactivation.

We have shown that NK cells from viremic and VS HIV+ individuals are phenotypically activated and degranulate spontaneously (28). Our observations of increased NK cell CD38 expression in viremic HIV+ individuals have been confirmed (44) and associated with HIV disease progression. Significantly, our observation that NK activation is elevated in VS HIV+ individuals has been supported by evidence that it also occurs in elite controllers (44). We asked whether increased NK activation and degranulation could be explained by expansion of FcRγ− NK cells, but there was no difference in spontaneous degranulation. Contrary to expectation, FcRγ− NK cells expressed lower levels of CD38 and HLA-DR than did conventional NK cells.

We have shown that NK cells from viremic and VS HIV+ individuals were incubated with Raji (top panel) or rituximab-opsonized Raji (bottom panel) cells at an E:T ratio of 1:1. CD107a expression on both FcRγ− and conventional (FcRγ+) CD56dim NK cells was measured as described in Materials and Methods. (B) Freshly sampled whole blood from seven VS HIV+ individuals (additional to those described in Table I) was incubated with a pool of overlapping HIV envelope peptides and serum from a single ADCC responder as described in Materials and Methods and then surface expression of CD107a and intracellular IFN-γ levels in both FcRγ− and conventional NK cells was measured by flow cytometry. Top panels show the contour plots from a representative donor indicating CD107a (left) and IFN-γ expression (right) versus FcRγ. Aggregate data for all seven donors are shown in the bottom panels. (C) Whole blood from 10 HIV+ donors was incubated for 4 h at 37°C then CD107a expression was measured in FcRγ− and conventional (FcRγ+) CD56dim NK cells as described in Materials and Methods. In the left panel, a comparison of CD107a expression on total CD56dim NK cells between HIV− and HIV+ donors is shown. In the right panel, CD107a expression on FcRγ− and conventional (FcRγ+) CD56dim NK cells from VS HIV+ donors is shown. (D) CD38 and HLA-DR expression in FcRγ− and conventional NK cells was measured in whole blood collected from eight VS HIV+ donors. HLA-DR versus CD38 contour plots from FcRγ− (red) and conventional (gray) CD56dim NK cells from a representative donor are shown in the left panel, and CD38 expression in the two populations from all donors is shown in the right panel. Differences between HIV− and HIV+ donors (C) were assessed by a Mann–Whitney U test. Comparisons between FcRγ− and conventional NK cells were made using a Wilcoxon signed rank test. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 4.** (A) PBMC prepared from eight VS HIV+ individuals (median age [range] = 52.5 [34–70] y), chosen because they exhibited ∼50% FcRγ− CD56dim NK cells, were incubated with Raji (top panel) or rituximab-opsonized Raji (bottom panel) cells at an E:T ratio of 1:1. CD107a expression on both FcRγ− and conventional (FcRγ+) CD56dim NK cells was measured as described in Materials and Methods. (B) Freshly sampled whole blood from seven VS HIV+ individuals (additional to those described in Table I) was incubated with a pool of overlapping HIV envelope peptides and serum from a single ADCC responder as described in Materials and Methods and then surface expression of CD107a and intracellular IFN-γ levels in both FcRγ− and conventional NK cells was measured by flow cytometry. Top panels show the contour plots from a representative donor indicating CD107a (left) and IFN-γ expression (right) versus FcRγ. Aggregate data for all seven donors are shown in the bottom panels. (C) Whole blood from 10 HIV+ donors was incubated for 4 h at 37°C then CD107a expression was measured in FcRγ− and conventional (FcRγ+) CD56dim NK cells as described in Materials and Methods. In the left panel, a comparison of CD107a expression on total CD56dim NK cells between HIV− and HIV+ donors is shown. In the right panel, CD107a expression on FcRγ− and conventional (FcRγ+) CD56dim NK cells from VS HIV+ donors is shown. (D) CD38 and HLA-DR expression in FcRγ− and conventional NK cells was measured in whole blood collected from eight VS HIV+ donors. HLA-DR versus CD38 contour plots from FcRγ− (red) and conventional (gray) CD56dim NK cells from a representative donor are shown in the left panel, and CD38 expression in the two populations from all donors is shown in the right panel. Differences between HIV− and HIV+ donors (C) were assessed by a Mann–Whitney U test. Comparisons between FcRγ− and conventional NK cells were made using a Wilcoxon signed rank test. *p < 0.05, **p < 0.01, ***p < 0.001.
expression of CD38 in FcRγ2 NK cells may reflect decreased chaperone function. Continued activation of NK cells in VS and elite controller HIV+ individuals adds to the monocyte activation data we have published (2, 3) demonstrating that the innate immune system is activated in these individuals.

Loss of NKp30 and NKp46 expression has been reported in HIV infection (47–49) but the mechanism underlying their loss is not known. Our data suggest that expansion of FcRγ2 NK cells in HIV+ individuals contributes to this loss and that it persists despite successful cART. Although NKp30 associates with TCRζ (50) and signals via TCRζ-dependent mechanisms (51), our data are consistent with a role for FcRγ as a molecular chaperone for NKp30. Similarly, NKp46 associates with TCRζ (52) but does not require it for surface expression (53), which is consistent with a role for FcRγ as the molecular chaperone for this receptor. NKp30 can mediate recognition and promote killing of fungal pathogens that cause AIDS-defining illnesses such as *Cryptococcus* and *Candida* spp. (54). Thus, expansion of FcRγ− NK cells in viremic HIV patients with low CD4 T cell counts may increase the susceptibility of such individuals to fungal opportunistic infections, although the persistence of these cells does not appear to maintain susceptibility to fungal infections on cART.

In summary, a unique subset of FcRγ− NK cells associated with CMV Ab levels in the general population is expanded in HIV-infected individuals and can represent up to 90% of CD56dim NK cells. In chronically infected HIV+ individuals their expansion correlates with plasma markers of innate immune activation. The long-term health consequences of expansion of FcRγ− NK cells lacking NKp30 and NKp46 need to be determined. Given the importance of these receptors for immunosurveillance of tumors,
the population may contribute to increased incidence of cancer in HIV+ patients receiving cART (55).

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Disclosures
The authors have no financial conflicts of interest.

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