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Virologically Suppressed HIV Patients Show Activation of NK Cells and Persistent Innate Immune Activation

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FcγR is an ITAM-containing adaptor required for CD16 signaling and function in NK cells. We have previously shown that NK cells from HIV patients receiving combination antiretroviral therapy (cART) have decreased FcγR expression, but the factors causing this are unknown. We conducted a cross-sectional study of a cART-naive viremic patients (ART−), virologically suppressed patients receiving cART (ART+), and HIV-uninfected controls. CD8+ T cells were activated, as assessed by CD38HLA-DR expression, in ART− patients (p < 0.0001), which was significantly reduced in ART+ patients (p = 0.0005). In contrast, CD38HLA-DR+ NK cells were elevated in ART− patients (p = 0.0001) but did not decrease in ART+ patients (p = 0.88). NK cells from both ART− and ART+ patients showed high levels of spontaneous degranulation in ex vivo whole blood assays as well as decreased CD16 expression (p = 0.0001 and p = 0.0025, respectively), FcγR mRNA (p < 0.0001 for both groups), FcγR protein expression (p = 0.0016 and p < 0.0001, respectively), and CD16-dependent Syk phosphorylation (p = 0.0001 and p = 0.003, respectively). HIV-infected subjects showed alterations in NK activation, degranulation, CD16 expression and signaling, and elevated plasma markers of inflammation and macrophage activation, that is, neopterin and sCD14, which remained elevated in ART+ patients. Alterations in NK cell measures did not correlate with viral load or CD4 counts. These data show that in HIV patients who achieve viral suppression following cART, NK cell activation persists. This suggests that NK cells respond to factors different from those driving T cell activation, but which are associated with inflammation in HIV patients. The Journal of Immunology, 2012, 189: 000–000.

Combination antiretroviral therapy (cART) has reduced the incidence of AIDS in HIV-infected individuals; however, cART cannot eliminate HIV from the body, and HIV infection is considered a chronic viral disease. HIV-infected individuals receiving cART continue to have a significantly shorter life expectancy, even in settings of advanced medical care (1), and they experience higher rates of non-AIDS comorbidities such as cardiovascular disease and non-AIDS cancers (2–5), many of which have an inflammatory etiology and may be related to continuing immunodeficiency (5). The success in reconstituting a functional immune system, defined by restoration of CD4 T cell counts following cART, is limited by immune activation and associated with pre-cART endotoxemia (6, 7).

Immune activation in HIV patients receiving cART is thought to be driven in part by endotoxemia resulting from a compromise in immunity due to destruction of T cells in GALT (8). Immune activation may also be caused by residual HIV viremia, at levels undetectable by standard clinical assays (1–30 copies/ml), which persists for at least 6 y in most HIV patients receiving cART (9). GALT integrity is not restored in patients receiving cART for up to 7 y, and persistent T cell loss in GALT is associated with increased T cell activation (10, 11). The effect of persistent immune activation on the innate immune system is poorly understood.

We have previously reported changes in NK cells purified from HIV patients receiving cART, where expression of key signaling proteins required for Ab-dependent cellular cytotoxicity (ADCC) was substantially reduced (12). NK cells in peripheral blood are commonly defined as belonging to one of two subsets. A minor CD56brightCD16− subpopulation produces high quantities of cytokines, including IFN-γ, in response to stimuli such as monocyte-derived IL-12 (13). The major CD56dimCD16+ subset is more efficient at killing virus-infected or transformed host cells, and the expression of the Fcγ receptor IIIA, CD16, on this subset enables it to perform ADCC. Signaling through the CD16 receptor, triggered by engaging IgG-opsonized targets, results in the release of cytotoxic granules containing perforin and granzymes by CD56dimCD16+ NK cells (14). ADCC signaling depends absolutely on the phosphorylation of ITAMs present on the CD16 intracellular adaptor protein FcγR (15). This allows recruitment of spleen tyrosine kinase, Syk, to the CD16 complex, which is subsequently activated and promotes downstream signaling required for exocytic degranulation (14, 16). The CD16 molecule, however,
can also associate with the TCR ζ-chain (TCRζ or CD247), a functional homolog of FcRγ (17). TCRζ can form heterodimers with FcRγ (18); however, at least in murine NK cells, these heterodimers do not appear to associate with CD16 or act as a CD16 chaperone (19).

We have previously shown that expression of FcRγ in NK cells is decreased in HIV-infected individuals receiving cART compared with HIV-uninfected controls (12). We hypothesized that HIV-associated immune activation causes downregulation of FcRγ in NK cells, leading to inhibition of ADCC signaling. In this study, we determine the relationship between NK activation, decreased FcRγ expression, CD16 surface expression, intracellular signaling, and, as a surrogate for ADCC degranulation activity, CD107a mobilization (16). We show that NK cells remain activated in virologically suppressed HIV-infected patients receiving cART and that defective NK cell ADCC signaling is not restored by cART. Importantly, NK cell activation and defective ADCC signaling correlate with plasma and cellular markers of immune activation and inflammation, but not with HIV RNA or CD4 counts.

Materials and Methods

Study groups

HIV-1–infected male patients were recruited following approval by the Ethics Committee of The Alfred Hospital. The inclusion criterion was: HIV Ab+ (>18 y age. Exclusion criteria were: immunomodulatory therapy (IL-2, hydroxyurea or prednisolone, HIV therapeutic vaccine), hepatitis B virus, and/or hepatitis C virus RNA or Ab+ or autoimmune disease. Patients were assigned to either the treatment-naive group (ART−) or, if they were currently receiving cART, to the on-treatment group (ART+). HIV-1–uninfected controls, self reporting as being healthy, were age- and sex-matched.

Cell phenotyping

Whole blood was incubated with the following Abs for 30 min, 22°C: CD3-FITC, CD4-PE-Cy7, CD8-V450, CD16-PE-Cy7, CD38-PE, HLA-DR-PerCP-Cy5.5. Cells were then washed and resuspended in cold FACS buffer (PBS−, 2 mM EDTA, 1% newborn calf serum [HyClone]). CD3+CD56+ NK cells were sorted and washed once in cold PBS−. Cells (2 × 10^6) from each population were lysed in 350 μL RNA lysis/binding buffer (100 mM Tris-HCl, 500 mM LiCl, 10 mM EDTA, 5 mM DTT, 1% LDS). The remaining cells were lysed in 20–50 μL 2.5% Triton X-100–based lysis buffer and snap frozen in liquid nitrogen prior to protein analysis.

mRNA extraction and quantitative real-time PCR

RNA was purified using magnetic bead separation (30 μL RNA beads; GentoPrep) and reverse transcribed using a primer first strand cDNA synthesis kit (Roche) according to the manufacturer’s protocol. Quantitative real-time PCR was performed using Roche FastStart Universal SYBR Green Master Mix and a Stratagene MX3000P instrument. Primers were designed to allow identical thermal profiles for each product (10 min at 95°C, 40 cycles of 20 s at 95°C, 20 s at 62°C, and 40 s at 72°C, and 1 min at 95°C). The following primer sets were used (including GenBank accession codes, available at http://www.ncbi.nlm.nih.gov/nuccore/): FcRγ, forward, 5′-GGTTCGACGCTGTTGCTGTC-3′, reverse, 5′-CCTCTGCACATGGATGCTTC-3′ (accession no. M33196); TCRζ, forward, 5′-AGTTCAGGACGGCCAGAC-3′, reverse, 5′-ACAGCGCTTCTGAGGTTC-3′ (accession no. DQ07217); DAP12, forward, 5′-GATGGGAGACCTGGACG-3′, reverse, 5′-ACAGCTGGTCGACCTG-3′ (accession no. AF019563); GAPDH, forward, 5′-AGTCCACTGGCCGCTTACC-3′, reverse, 5′-AGCAGTTGGTGCTGAGGAG-3′ (accession no. AT34084); CD16, forward, 5′-TGAGGTCTGAGCAGTGGAG-3′, reverse, 5′-GGGTGACACTGCAAAACCTT-3′ (accession no. NM_00127596). mRNA levels were quantified using the comparative threshold method, with GAPDH mRNA as internal standard.

Protein extraction and immunoblotting

Protein extracts were incubated for 20 min on ice, followed by 5 min at 37°C and then centrifuged at 20,800 × g for 4°C for 10 min. Ten micrometers of protein per sample was denatured at 95°C for 10 min and separated using 10% Bis-Tris Midi Gels (Invitrogen), and NuPAGE MES SDS running buffer. Proteins were transferred onto a polyvinylidene difluoride membrane using an iBlot dry blotting system (Invitrogen). Membranes were blocked with 5% skimmed milk in TBS plus 0.1% Tween 20 for 30 min and probed with mouse anti-GAPDH (Santa Cruz Biotechnology) plus rabbit anti-FcRγ polyclonal serum (gift of Mark Hogarth, Burnet Institute) at 4°C overnight. After three washes with TBS plus 0.1% Tween 20, membranes were incubated with secondary Ab (goat anti-mouse Alexa Fluor 680 plus goat anti-rabbit Alexa Fluor 750; Molecular Probes) for 2 h at room temperature. Fluorescence was quantified using an Odyssey infrared imager (LI-COR Biosciences).

Measurement of soluble CD14, soluble CD163, and neopterin

Soluble (s)CD14, sCD163, and neopterin levels were measured in plasma by using the Quantikine human sCD14 kit (R&D Systems, Minneapolis, MN), human sCD163 kit (IQ Products, Groningen, The Netherlands), and the human sCD14 kit (R&D Systems). sCD14, sCD163, and neopterin were quantified in plasma using the Quantikine human sCD14 kit (R&D Systems) according to the manufacturer’s protocol. Plasma samples were diluted 1:400 (sCD14), 1:500 (sCD163), and 1:4 (neopterin).

Measurement of plasma LPS and 16S genomic DNA in plasma

Plasma levels of LPS and genomic bacterial DNA encoding the 16s rRNA gene were measured as described in Kramski et al. (20).

Table I. Study population

<table>
<thead>
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<th></th>
<th>HIV−</th>
<th>HIV+</th>
<th>HIV+</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>n/a</td>
<td>Naive</td>
<td>cART</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>16</td>
<td>17</td>
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<tr>
<td>Age, y</td>
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<td>40 (26–63)</td>
<td>49 (28–65)</td>
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<tr>
<td>% CD4+ cells</td>
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<td>31 (4.57–49.7)</td>
<td>34 (15.3–53.6)</td>
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<td>600 (129–1199)</td>
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<tr>
<td>Nadir CD4+</td>
<td>379.5 (8–546)</td>
<td>208 (14–403)</td>
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<tr>
<td>Viral load</td>
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<td>Years on ART</td>
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<td>11.5 (0.25–18)</td>
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<tr>
<td>Years of virologic suppression</td>
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<td>n/a</td>
<td>2.9 (0.6–7.7)*</td>
</tr>
</tbody>
</table>

All values are given as medians and ranges. *Data for n = 16 patients; 1 patient in the ART+ group had a detectable viral load (1700). n/a, Not applicable.
FIGURE 1. NK cell activation phenotype and plasma markers of myeloid activation are not decreased in virologically suppressed HIV patients. (A) Representative dot plots showing gating of CD4 and CD8 T cells, CD56dimCD16+ NK cells, and CD56brightCD16- NK cells and their coexpression of CD38 and HLA-DR in a HIV-uninfected control (HIV-), cART-naive HIV-infected patient (ART-), and virologically suppressed HIV-infected individual receiving cART (ART+). Lymphocytes were initially gated in forward and side scatter plots and then events were analyzed on the dot plots as shown. (B) Comparison of the proportion of CD38+HLA-DR+ double-positive activated lymphocytes between HIV-uninfected controls and HIV-infected subjects stratified for treatment status. KW, Kruskal–Wallis p value). (C) Comparison of levels of plasma markers of myeloid cell activation between HIV-uninfected controls and HIV-infected subjects stratified for treatment status. Differences between groups were assessed using a Kruskal–Wallis test followed by serial Wilcoxon signed rank sum (post) tests. Statistical significance was assumed after adjusting for multiple comparisons when \( p < 0.017 \).
FIGURE 2. NK cell degranulation and CD16 expression are not returned to control levels in virologically suppressed HIV patients. (A) CD56<sup>dim</sup>CD16<sup>+</sup> NK cells present within whole blood were gated as shown in the upper panels. Representative dot plots showing isotype control (middle panels) and CD107a (lower panels) staining within the CD3<sup>+</sup>CD56<sup>dim</sup> gate measured after 3 h incubation are shown for an HIV<sup>−</sup> (middle left and lower left panels; both plots showing the 5% isotype gate) and ART<sup>+</sup> individual (middle right and lower right panels; both plots showing the 5% isotype gate). (B) Comparison of the proportion of CD56<sup>dim</sup>CD16<sup>+</sup> NK cells undergoing degranulation in HIV-uninfected and HIV-infected subjects stratified for treatment status. Comparison of CD16 surface expression (C) and mRNA expression (D) on CD56<sup>dim</sup>CD16<sup>+</sup> NK cells. (E) Correlation of CD16 surface and mRNA expression in HIV-uninfected subjects (○), HIV-infected treatment naive (●), and HIV-infected patients currently receiving treatment (◆). Differences between groups were assessed using a Kruskal–Wallis test followed by serial Wilcoxon signed rank sum (post) tests. Statistical significance was assumed after adjusting for multiple comparisons when \( p < 0.017 \). Correlations were assessed using a Spearman rank correlation; regression line and 95% confidence intervals are shown.
**Results**

NK cell activation is not reduced by ART

We recruited 33 male HIV patients of whom 16 were ART\(^-\) and 17 were ART\(^+\) at enrollment. Twenty age-matched male HIV-uninfected subjects were recruited as controls (Table I). The ART\(^+\) patients received antiretroviral therapy for a median time of 11.5 y (range, 0.25–18 y) and had a sustained undetectable viral load for a median time of 2.9 y (range, 0.6–7.7 y) prior to recruitment. We measured cellular markers of immune activation (CD38 and HLA-DR coexpression) on CD56\(^\text{dim}\) and CD56\(^\text{bright}\) NK cells, as well as CD4 and CD8 T cells, using whole blood phenotyping assays (Fig. 1). Representative dot plots for a member of each study group are shown in Fig. 1A. As reported by others (21, 22), both CD4 and CD8 cells showed increased CD38/HLA-DR expression in ART\(^+\) patients (p = 0.0007 and p < 0.0001, respectively), which was significantly decreased in ART\(^-\) patients (p = 0.01 and p = 0.0005, respectively) (Fig. 1B). CD4 T cell activation returned to baseline levels in ART\(^+\) patients compared with controls, but CD8 T cell activation did not (p = 0.0008). CD38/HLA-DR coexpression on CD56\(^\text{dim}\)/CD16\(^+\) NK cells, which in fact reflects changes in HLA-DR expression as NK cells constitutively express CD38, was also significantly increased in ART\(^-\) patients (p = 0.0001). Surprisingly, and in contrast to T cells, HLA-DR coexpression on NK cells was not reduced following suppression of viremia with ART (p = 0.88). We did not observe significantly altered expression of HLA-DR in the CD56\(^\text{dim}\)/CD16\(^+\) NK cell subpopulation (Kruskal–Wallis p = 0.12). We also did not observe significantly altered expression of HLA-DR in the CD56\(^\text{dim}\)/CD16\(^+\) NK cell subpopulation (Kruskal–Wallis p = 0.14), which are already more highly activated as indicated by higher levels of HLA-DR expression compared with the CD56\(^\text{dim}\)/CD16\(^+\) NK cells (median HLA-DR\(^+\) cells, 16 [HIV\(^-\)], 23 [ART\(^-\)], and 34% [ART\(^+\)]). We next measured markers of inflammation and myeloid cell activation in the patient groups. Concentrations of sCD14, neopterin, and sCD163 were elevated in plasma from both ART\(^-\) and ART\(^+\) patients compared with controls (sCD14, p < 0.0001 for both ART\(^-\) and ART\(^+\); neopterin, p < 0.0001 [ART\(^-\)] and p = 0.019 [ART\(^+\)]; sCD163, p < 0.0001 [ART\(^-\)] and p = 0.0012 [ART\(^+\)]; Fig. 1C). There was no significant difference in plasma sCD14 or neopterin between ART\(^-\) and ART\(^+\) patients (p = 0.16 and p = 0.15, respectively). Thus, these plasma markers of inflammation and myeloid activation showed a similar pattern of increase as does NK cell activation but not T cell activation.

To further examine the activation status of NK cells in HIV patients, we measured their rate of degranulation in whole blood by measuring the mobilization of CD107a in the absence of exogenous targets. CD56\(^\text{dim}\) NK cells in whole blood were gated as shown (Fig. 2A, upper panels), and transient surface expression of CD107a was measured after a 3-h incubation in the absence of added targets. To objectively compare measurements from all study groups, CD107a expression was obtained based on a 5% isotype gate as an internal standard (representative dot plots from HIV-infected and HIV-uninfected donors are shown in Fig. 2A, lower panels). Differences in medians between HIV\(^-\), ART\(^-\), and ART\(^+\) groups were tested for significance using the Kruskal–Wallis test followed by serial Wilcoxon rank sum (post) tests. Statistical significance was assumed when p < 0.05 or, for Wilcoxon rank sum tests comparing three groups, when p < 0.017. Analyses were performed using Stata (Mac v10.1; StataCorp, College Station, TX).

**FIGURE 3.** Expression of the signaling adaptor proteins FcR\(\gamma\), TCR\(\zeta\), and DAP12 are not returned to control levels in virologically suppressed HIV patients. Comparison of FcR\(\gamma\) mRNA (A) and protein (B) expression in CD56\(^\text{dim}\)/CD16\(^+\) NK cells in HIV-uninfected controls and HIV-infected subjects stratified for treatment status. (C) Correlation of FcR\(\gamma\) mRNA and protein expression in HIV-infected patients (●, ART\(^-\); ○, ART\(^+\)). Comparison of TCR\(\zeta\) (D) and DAP12 (E) mRNA expression in CD56\(^\text{dim}\)/CD16\(^+\) NK cells in HIV-uninfected controls and HIV-infected subjects stratified for treatment status. Differences between groups were assessed using a Kruskal–Wallis test followed by serial Wilcoxon signed rank-sum (post) tests. Statistical significance was assumed after adjusting for multiple comparisons when p < 0.017. Correlations were assessed using a Spearman rank correlation; regression line and 95% confidence intervals are shown.
middle and lower panels; gates represent a 5% isotype gate). NK cells from HIV-infected individuals had elevated CD107a mobilization in the absence of exogenous targets, irrespective of their cART status \((p = 0.0002 \text{ [ART}^- \text{]} \text{ and } p = 0.013 \text{ [ART}^+ \text{]; Fig. 2B})\) compared with HIV-uninfected individuals. Activation of NK cells in HIV-infected individuals was associated with decreased surface CD16 expression on CD56dimCD16+ NK cells, which was again independent of treatment status, with both ART- and ART+ patients showing significant reduction \((p = 0.0001 \text{ [ART}^- \text{]} \text{, } p = 0.0025 \text{ [ART}^+ \text{]; Fig. 2C})\). The extent of degranulation inversely correlated with CD16 surface expression on CD56dimCD16+ NK cells \((\text{Spearman rho} = -0.51, p = 0.0019)\). HIV infection was also associated with decreased CD16 mRNA expression in both ART- and ART+ patient groups, indicating that at least part of the reduction of CD16 expression may be due to decreased transcription \((p = 0.025 \text{ [ART}^- \text{] } \text{, } p = 0.0026 \text{ [ART}^+ \text{]; Fig. 2D})\). That CD16 surface expression is regulated in part by transcription is supported by the significant correlation of CD16 surface expression \(\text{(mean fluorescence intensity)}\) and mRNA levels \((\text{Spearman rho} = 0.39, p = 0.015; \text{Fig. 2E)}}\).

**CD16 surface expression is modulated in HIV-infected individuals in part by reduced transcription and expression of the chaperone FcR\(_\gamma\)**

Because CD16 surface expression was decreased in virologically suppressed HIV patients receiving cART, we next measured FcR\(_\gamma\) expression, which acts as both a chaperone for CD16 surface protein expression and as an indispensable transducer of CD16 signaling. NK cells were sorted from patient blood by flow cytometry, and aliquots of purified cells were extracted for protein and RNA preparation. Expression of FcR\(_\gamma\) mRNA \((\text{Fig. 3A)}}\) and protein \((\text{Fig. 3B})\) was significantly decreased in both ART- and ART+ patients compared with HIV-uninfected controls \((\text{mRNA}, p < 0.0001 \text{ for both ART}^- \text{ and ART}^+ \text{; protein}, p = 0.0016 \text{ for ART}^- \text{ and } p < 0.0001 \text{ for ART}^+)\). Significantly, there was no difference in FcR\(_\gamma\) expression between ART- and ART+ patients \((\text{mRNA}, p = 0.14; \text{protein}, p = 0.72)\). In HIV patients, FcR\(_\gamma\) mRNA levels correlated strongly with FcR\(_\gamma\) protein expression \((\text{Spearman rho} = 0.81, p < 0.0001; \text{Fig. 3C})\). These data demonstrate that FcR\(_\gamma\) protein expression is reduced in HIV-infected individuals at a transcriptional level and is not restored by cART.

Because other ITAM-containing signaling adaptor proteins may potentially compensate for loss of FcR\(_\gamma\), we investigated expression of the adaptor proteins TCR\(\zeta\) and DAP12. Expression of TCR\(\zeta\) mRNA \((\text{Fig. 3D})\) and DAP12 \((\text{Fig. 3E})\) were decreased in both ART- and ART+ patients compared with HIV-uninfected controls \((\text{TCR}\zeta, p = 0.0012 \text{ for ART}^- \text{ and } p < 0.0001 \text{ for ART}^+; \text{DAP12, } p = 0.031 \text{ for ART}^- \text{ and } p = 0.0013 \text{ for ART}^+)\). There was no difference in TCR\(\zeta\) or DAP12 expression between ART+ and ART- patients \((\text{TCR}\zeta, p = 0.53; \text{DAP12, } p = 0.12)\).

To investigate the functional consequences of FcR\(_\gamma\) and TCR\(\zeta\) downregulation, signal transduction through CD16 was measured using a whole blood Phosflow assay to quantify CD16-dependent ZAP70/Syk phosphorylation in NK cell subsets. As part of this assay, we cross-linked surface CD16 on CD56dimCD16+ NK cells in whole blood by sequentially adding anti-CD16 primary and goat anti-mouse secondary Abs, as described in detail in Lichtfuss et al. (16).

CD56dim and CD56bright NK cells were gated and separately analyzed as shown in Fig. 4A \(\text{(left and middle panels)}\). In agree-

**FIGURE 4.** CD16 signaling is not returned to control levels in virologically suppressed HIV patients. (A) Representative histogram showing Phosflow analysis of CD16-dependent Syk/ZAP70 phosphorylation. The approximate position of lymphocytes (L), monocytes (M), and neutrophils (N) are shown in the forward versus side scatter profile (left panel). Events within the lymphocyte gate were plotted on a CD3 versus CD56 profile to define the CD56dim and CD56bright NK cell subsets (middle panel). Histogram overlay depicts isotype control Ab staining (dotted line), unstimulated baseline control staining (dashed line), Syk/ZAP70 phosphorylation in CD56dim cells from an HIV-uninfected subject (light gray histogram), and an ART- HIV-infected subject (dark gray histogram). The proportion of percentage positive cells was calculated in comparison with baseline using a population comparison algorithm. Comparison of Syk/ZAP70 phosphorylation measured 1.5 min after CD16 cross-linking \(\text{(B)\) and at baseline \(\text{(C)}.\) Differences between groups were assessed using a Kruskal–Wallis test followed by serial Wilcoxon signed rank-sum (post) tests. Statistical significance was assumed after adjusting for multiple comparisons when \(p < 0.017).
NK cell activation and loss of CD16 signaling are not associated with CD4 count or viral load

To determine which factors may cause NK cell activation and loss of CD16 signaling, we initially correlated these parameters with traditional measures of HIV-1 disease progression. In viremic HIV-infected individuals, there was no correlation between NK activation (determined either as the proportion of CD38\(^+\)HLA-DR\(^+\) NK cells or NK cell degranulation) with either CD4 counts or viral load (Table II). In contrast, both CD4 and CD8 T cell activation correlated with viral load as reported by others (23). In the combined group of ART\(^-\) and ART\(^+\) patients, neither NK cell nor T cell activation correlated with CD4 counts. A similar lack of correlation of CD16 expression with either viral load or CD4 count was observed with CD16 expression on CD56\(^{dim}\) CD16\(^+\) NK cells.

We did not observe any correlation between NK cell or T cell activation and either plasma levels of sCD14, neopterin, endotoxin, or bacterial 16S RNA levels in plasma (Table II).

Discussion

We show that NK cells remain activated in patients receiving cART who have received cART for a median duration of 11.5 y (range, 0.25–18 y) and maintained an undetectable viral load for a median time of 2.9 y (range, 0.6–7.7 y). This contrasts with T cell activation, which is reduced by cART in the same group of patients. We also show that activation of NK cells in HIV patients is associated with impaired CD16 signaling owing to loss of CD16 and expression of its chaperone and signaling proteins FcR\(\gamma\) and TCR\(\gamma\). Significantly, NK cell activation, decreased FcR\(\gamma\) expression, and impaired signaling did not correlate with HIV RNA or CD4\(^-\) T cell counts. Time of undetectable viral load in patients receiving cART did not correlate with measures of the study endpoint FcR\(\gamma\) protein levels (n = 11, Spearman rho = 0.091, p = 0.79), with surface expression of CD16 (n = 8, Spearman rho = −0.32, p = 0.41), or with HLA-DR expression (n = 9, Spearman rho = −0.067, p = 0.86) on CD56\(^{dim}\) NK cells. However, both HIV-infected study groups showed NK cell activation and also exhibited loss of CD16 signaling and increased levels of plasma neopterin, sCD14, and sCD163, which suggests that these events are linked to myeloid activation and hence activation of the innate immune system. The different responses of NK and T cell activation to cART suggest that they are activated by different factors in HIV patients. Our data suggest that current cART regimens are unable

| Table II. Correlates of NK cell and T cell parameters with HIV disease in HIV-infected subjects |
|-----------------|-------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | CD4 Count | Viral Load | Neopterin | sCD14 | FcR\(\gamma\) | HLA-DR | CD16 | CD56 | LPS |
| NK cells | CD16 surface expression, MFI | Spearman rho = 0.0083, p = 0.79 (12) | Spearman rho = -0.26, p = 0.19 (20) | Spearman rho = 0.26, p = 0.17 (20) | Spearman rho = 0.016, p = 0.51 (11) | Spearman rho = 0.81, p = 0.016 (11) | Spearman rho = 0.22, p = 0.12 (21) | Spearman rho = 0.6, p = 0.11 (11) |
|          | CD16 expression | Spearman rho = 0.0083, p = 0.79 (12) | Spearman rho = -0.26, p = 0.19 (20) | Spearman rho = 0.26, p = 0.17 (20) | Spearman rho = 0.016, p = 0.51 (11) | Spearman rho = 0.81, p = 0.016 (11) | Spearman rho = 0.22, p = 0.12 (21) | Spearman rho = 0.6, p = 0.11 (11) |
|          | FcR\(\gamma\) mRNA expression | Spearman rho = 0.0083, p = 0.79 (12) | Spearman rho = -0.26, p = 0.19 (20) | Spearman rho = 0.26, p = 0.17 (20) | Spearman rho = 0.016, p = 0.51 (11) | Spearman rho = 0.81, p = 0.016 (11) | Spearman rho = 0.22, p = 0.12 (21) | Spearman rho = 0.6, p = 0.11 (11) |
|          | LPS | Spearman rho = 0.0083, p = 0.79 (12) | Spearman rho = -0.26, p = 0.19 (20) | Spearman rho = 0.26, p = 0.17 (20) | Spearman rho = 0.016, p = 0.51 (11) | Spearman rho = 0.81, p = 0.016 (11) | Spearman rho = 0.22, p = 0.12 (21) | Spearman rho = 0.6, p = 0.11 (11) |
| CD16 | CD16 expression | Spearman rho = 0.0083, p = 0.79 (12) | Spearman rho = -0.26, p = 0.19 (20) | Spearman rho = 0.26, p = 0.17 (20) | Spearman rho = 0.016, p = 0.51 (11) | Spearman rho = 0.81, p = 0.016 (11) | Spearman rho = 0.22, p = 0.12 (21) | Spearman rho = 0.6, p = 0.11 (11) |
| CD16 dim | CD16 expression | Spearman rho = 0.0083, p = 0.79 (12) | Spearman rho = -0.26, p = 0.19 (20) | Spearman rho = 0.26, p = 0.17 (20) | Spearman rho = 0.016, p = 0.51 (11) | Spearman rho = 0.81, p = 0.016 (11) | Spearman rho = 0.22, p = 0.12 (21) | Spearman rho = 0.6, p = 0.11 (11) |
| CD56 | CD16 expression | Spearman rho = 0.0083, p = 0.79 (12) | Spearman rho = -0.26, p = 0.19 (20) | Spearman rho = 0.26, p = 0.17 (20) | Spearman rho = 0.016, p = 0.51 (11) | Spearman rho = 0.81, p = 0.016 (11) | Spearman rho = 0.22, p = 0.12 (21) | Spearman rho = 0.6, p = 0.11 (11) |
| CD56 bright | CD16 expression | Spearman rho = 0.0083, p = 0.79 (12) | Spearman rho = -0.26, p = 0.19 (20) | Spearman rho = 0.26, p = 0.17 (20) | Spearman rho = 0.016, p = 0.51 (11) | Spearman rho = 0.81, p = 0.016 (11) | Spearman rho = 0.22, p = 0.12 (21) | Spearman rho = 0.6, p = 0.11 (11) |
| CD56 dim | CD16 expression | Spearman rho = 0.0083, p = 0.79 (12) | Spearman rho = -0.26, p = 0.19 (20) | Spearman rho = 0.26, p = 0.17 (20) | Spearman rho = 0.016, p = 0.51 (11) | Spearman rho = 0.81, p = 0.016 (11) | Spearman rho = 0.22, p = 0.12 (21) | Spearman rho = 0.6, p = 0.11 (11) |

MFI, Mean fluorescence intensity.

Spearman rho, \(\rho\) and \(p\) values, with the number of subjects in parentheses.
to reduce NK cell activation to normal levels and that targeting inflammation, in addition to suppressing viremia, will be required to fully restore NK cell-mediated immune function in these patients.

One potential source of inflammation in HIV patients is chronic endotoxemia arising from impaired barrier function of the gut epithelium caused by destruction of intestinal T cells during acute HIV infection (8). Endotoxin levels are not decreased significantly when patients achieve virologic suppression with cART, and our modeling has shown that decades of cART treatment may be required to reduce endotoxemia in HIV patients (7). We did not observe any correlation, however, between NK cell activation and plasma endotoxin levels or levels of bacterial 16S DNA, which suggests that the relationship between bacterial products in blood and NK cell activation is not direct. A similar conclusion was reached in a study of HIV patients and patients with inflammatory bowel disease, although in this study, which measured activation by expression of the acute activation marker CD69, activation was decreased in patients receiving cART (24). One caveat, however, is that the LAL endotoxin assay is not robust when used for measuring bacterial products in plasma (20). Longitudinal studies are required to establish whether NK activation and function are restored to normal levels after a sufficient duration of suppressive cART, which may also give indications about causality behind the associations we have observed.

HIV-infected individuals showed high basal degranulation of CD56dimCD16+ NK cells in whole blood incubated ex vivo, the levels of which were elevated in both ART− and ART+ patients relative to HIV-uninfected control subjects. This degranulation likely indicates heightened NK cell activity, which has been observed previously by others (25). Our observation that baseline levels of Syk/ZAP70 phosphorylation were not increased in HIV-infected individuals suggests that elevated degranulation is not likely a consequence of high levels of ADCC. We hypothesize that degranulation is due to the presence of targets for NK cell-mediated natural cytotoxicity, which we and others have shown is independent of Syk phosphorylation (16, 26). The existence and nature of these targets remains to be established. It has been shown that HIV-infected T cell blasts are killed by NK cells and induce ADCC via activation of NK cell inhibitory receptors through HLA-C and HLA-E expression (27); however, these cells are unlikely to be a significant cause of ADCC loss in virologically suppressed patients receiving cART due to their low prevalence. In contrast, endotoxin, which is elevated in HIV-infected individuals irrespective of cART, is known to upregulate NKG2D ligands such as MIC-A on monocytes (28). Activation of NKG2D is known to induce cross-tolerance, which results in loss of CD16 expression and several ITAM proteins (29).

In HIV-infected individuals, decreased expression of CD16 and its chaperone/signaling adaptor FcRγ and signaling adaptor TCRζ resulted in loss of CD16-dependent signaling that is essential for ADCC. Loss of CD16 has been shown to be partially dependent on matrix metalloproteinase induction, which promotes shedding of the extracellular domain of the receptor (30). Our data showing a direct correlation of CD16 mRNA expression with CD16 surface expression indicate that an additional mechanism regulating CD16 surface expression at a transcriptional level is operating in chronic HIV patients. Whether matrix metalloproteinase activity also has an effect on the expression of the FcRγ chaperone is not clear. Our finding of reduced CD16 and FcRγ mRNA levels in HIV patients implies that additional mechanisms underlie decreased expression of ADCC receptors on NK cells. Furthermore, the association of FcRγ expression with CD16 surface expression suggests that the reduced expression of the chaperone is a significant factor.

HIV infection is accompanied by the accumulation of CD56−CD16+ NK cells (31), which may account for early reports of decreased numbers of NK cells in HIV-infected patients (25, 32). CD56−CD16+ NK cells are anergic (reviewed in Ref. 32) but retain the ability to synthesize and secrete MIP-1β (25), showing that NK cells lose function in a hierarchical manner in the setting of chronic viral infection. Because we gated specifically on CD3−CD56+ cells in the present study, the decreased expression of signaling molecules that we observed in HIV patients was not due to an increased proportion of CD56+ cells. It is likely that CD56−CD16+ cells arise from maturation of CD56dimCD16+ NK cells. It will be of interest to determine whether CD56+ NK cells expressing low levels of FcRγ, TCRζ, and DAP12 represent a precursor stage toward losing CD56, similar to the reduction of SIGLEC-7 expression (33, 34). Consistent with this concept, we have also observed a decreased expression of CD56 (data not shown) in addition to low CD16 expression within CD56+ NK cells from HIV-infected patients both on and off therapy.

Despite its absolute requirement for functional NK cell signaling (15), there have been no data published on FcRγ expression in HIV infection or other human diseases. However, reduced expression of TCRζ in T cells and NK cells has been documented in HIV-infected patients before the widespread use of ART (35). Limited data suggest a beneficial effect of ART on TCRζ expression in T cells (36); however, no increase in NK cell TCRζ or FcRγ expression at the mRNA level was detected in our ART+ group compared with ART− individuals. This also implies that FcRγ loss is not compensated by an increased expression of other homologous ITAM molecules, including DAP12, for which, to our knowledge, we show a pathological dysregulation for the first time in human disease. Whether this is associated with decreased function of NK cell receptors, which use DAP12 for signal transduction, needs to be investigated.

NK cells are able to lyse tumor- and virus-infected cells, but their potential role in preventing non-AIDS comorbidities in patients receiving ART has not been addressed. Non-AIDS cancers are an increasing cause of morbidity and mortality in HIV-infected persons, and continuing defects in NK cells may contribute to their incidence by failing to perform efficient surveillance. In this context, the sensitivity of ADCC signaling in NK cells to inflammation and immune activation suggests it may be a valuable marker determining the long-term consequences of HIV infection on patient morbidity and mortality after viremic suppression with ART.

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Disclosures
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References


