Terminal Effector CD8 T Cells Defined by an IKZF2^+IL-7R^- Transcriptional Signature Express FcγRIIIA, Expand in HIV Infection, and Mediate Potent HIV-Specific Antibody-Dependent Cellular Cytotoxicity

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HIV-1 infection expands large populations of late-stage differentiated CD8 T cells that may persist long after viral escape from TCR recognition. In this study, we investigated whether such CD8 T cell populations can perform unconventional innate-like antiviral effector functions. Chronic untreated HIV-1 infection was associated with elevated numbers of CD45RA^−CD57^− terminal effector CD8 T cells expressing FcγRIIIA (CD16). The FcγRIIIA^+ CD8 T cells displayed a distinctive transcriptional profile between conventional CD8 T cells and NK cells, characterized by high levels of IKZF2 and low expression of IL7R. This transcriptional profile translated into a distinct Nkp80^− IL-7Rx^c^ surface phenotype with high expression of the Helios transcription factor. Interestingly, the FcγRIIIA^+ CD8 T cells mediated HIV-specific Ab-dependent cellular cytotoxicity (ADCC) activity at levels comparable with NK cells on a per cell basis. The FcγRIIIA^+ CD8 T cells were highly activated in a manner that correlated positively with expansion of the CD8 T cell compartment and with plasma levels of soluble mediators of antiviral immunity and inflammation such as IP-10, TNF, IL-6, and TNFRII. The frequency of FcγRIIIA^+ CD8 T cells persisted as patients initiated suppressive antiretroviral therapy, although their activation levels declined. These data indicate that terminally differentiated effector CD8 T cells acquire enhanced innate cell-like characteristics during chronic viral infection and suggest that HIV-specific ADCC is a function CD8 T cells use to target HIV-infected cells. Furthermore, as the FcγRIIIA^+ CD8 T cells persist in treatment, they contribute significantly to the ADCC-capable effector cell pool in patients on antiretroviral therapy. The Journal of Immunology, 2019, 203: 2210–2221.

The exquisite sensitivity and specificity of TCR-mediated sensing of infection is central to the function of T cells but can also, in some situations, limit their ability to provide effective immunity. This is evident in the context of HIV-1 infection, in which the appearance of HIV-1–specific T cells coincides with initial viral decline; however, the response fails to completely suppress or clear infection (1−4). Since the initial characterization of HIV-specific cytotoxic CD8 T cells in the late 1980s (5−9), the limitations in their ability to control viral replication and clear infection are evident (10, 11). High mutation rates in

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Received for publication April 11, 2019. Accepted for publication August 20, 2019.

This work was supported by a cooperative agreement (W81XWH-07-2-0067) between the Henry M. Jackson Foundation for the Advancement of Military Medicine and the U.S. Department of Defense and with an Interagency Agreement (Y1-A1-26-42-07) with the Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, the Swedish Research Council, the Swedish Cancer Society, and the Karolinska Institutet. Sample collection for the Couples Observational Study was supported by the Bill and Melinda Gates Foundation (Grant 41185).

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The online version of this article contains supplemental material.

Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; ART, antiretroviral therapy; COS, Couples Observation Study; Eomes, eomesodermin; FDR, false discovery rate; HCV, hepatitis C virus; KIR, killer Ig-like receptor; MFI, mean fluorescence intensity; PCA, principal component analysis.

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HIV-1 contribute to the ability of the virus to escape adaptive T cell responses (3, 12–14). Also, HIV-specific T cells become functionally impaired during chronic infection, additionally limiting their ability to control viral replication (15–17). Indeed, polyfunctional HIV-specific T cell responses are associated with better disease outcomes compared with those with a narrower functional breadth (18–20). In chronic HIV-1 infection, the replicating viral quasispecies have, to a large extent, mutated away from the originally transmitted viral sequence under T cell selection pressure, and this probably contributes to the accumulation of late-stage effector CD8 T cells with a skewed maturational phenotype (21, 22).

Persistent pathogen replication in chronic infections, such as untreated HIV-1 infection, engages T cell–mediated immune responses continuously with sustained antigenic challenge. Interestingly, some chronic infections have been associated with expansion of an unusual subset of CD8 T cells expressing CD16 (23–25). CD16 is the low-affinity IgG Fc receptor and exists in two isoforms, FcγRIIIA (CD16a) and FcγRIIB (CD16b). CD16b is expressed exclusively by neutrophils and recognizes IgG-containing immune complexes, whereas CD16a is best characterized for its role in mediating Ab-dependent cellular cytotoxicity (ADCC) as a function of the innate immune system (26, 27, and reviewed in 28). NK cells are able to mediate a strong effector function in response to signaling through CD16-mediated stimulation. Whereas Fc receptors are generally not expressed by T cells, CD16 can sometimes be expressed by subsets of TCRαβ T cells (29–32). Growing evidence suggests the potential importance of ADCC in protection from HIV-1 infection (33, 34). Additionally, nonneutralizing Abs mediate an array of effector functions through their interactions with Fc receptors that may potentiate protection from HIV-1 infection or inhibit viral replication postinfection (35–40). Still, a better understanding of effector mechanisms, such as ADCC, involved in HIV-1 control is needed.

In this study, we hypothesized that late-stage differentiation of CD8 T cells may be associated with transcriptional changes that support innate-like effector functions in the T cell compartment. We demonstrate, in this study, that chronic, untreated HIV-1 infection is associated with the expansion of a late-stage differentiated CD8 T cell population expressing FcyRIIIA and that this population mediates HIV-specific ADCC. Furthermore, we show that the FcγRIIIA* CD8 T cells display a hybrid CD8 T cell and NK cell transcriptional profile characterized by high expression of NKP80 and the transcription factor Helios.

Materials and Methods

Patients and samples

Study participants aged 15–49 y were enrolled in a prospective community-based cohort to assess the prevalence and incidence of HIV-1 infection in Rakai District, Uganda, from 1998 to 2004 (Table I) (41–43). Infected subjects were identified between 1997 and 2002 with continued follow up through 2008. Blood samples from 103 randomly selected HIV-1 seropositive individuals and 40 community-matched seronegative controls were obtained: PBMCs were then isolated and cryopreserved as described previously (44). None of the patients had received antiretroviral therapy (ART). HIV-1 testing was performed as described previously (43). Positive samples were subjected to the Amplicor HIV-1 Monitor test, version 1.5 (Roche Diagnostics, Indianapolis, IN). The HIV-1–infected study participants initiating ART were from the Couples Observation Study (COS) in Kampala Uganda as previously described (45). The index partner in each HIV-1–serodiscordant couple was followed up after the initiation of ART. Samples were collected; CD4 T cell counts determined and viral load assessments made at baseline, 6 and 12 mo after initiation of ART.

Ethics statement

The study was approved by the following institutional review boards in the United States and Uganda: the institutional Review Boards of Uganda’s National Council for Science and Technology and the National AIDS Research Committee, as well as Division of Human Subjects Protection at the Walter Reed Army Institute of Research. All participants gave written-informed consent, or written-informed consent was obtained from the parent or legal guardian of those aged 17. For samples from the COS in Kampala, Uganda, all participants gave written-informed consent, and ethical approvals for the study were obtained from Uganda’s National Council for Science and Technology and the National AIDS Research Committee and the University of Washington.

Flow cytometry and mAbs

Cryopreserved specimens were thawed and washed. Counts and viability were assessed on the Guava PCA (Guava Technologies, Hayward, CA), using Guava ViaCount reagent. Standard flow cytometry phenotyping was performed as previously described (46). Commercial mAbs (clone) used in flow cytometry were as follows: CCR5/CD195 BV421 (2D7), CCR7/CD197 FITC (150503), CD144 allophycocyanin-H7 (M499), CD44 Alexa Fluor 700 (65E2), CD19 Alexa Fluor 700 (HIB19), CD16 allophycocyanin-Cy7, PE-Cy5, Pacific Blue and BV421 (568), CD161 PE-Cy5 (DX12), CD27 PerCP Cy5.5 (L128), PD-1/CD279 Alexa Fluor 647 and PE (EH12.1), CD3 AmCyan, allophycocyanin–H7, and PerCP-Cy5.5 (SK7), CD3 PE-CE594 (UCHT1), CD4 BV505 and allophycocyanin–H7 (SK3), CD38 allophycocyanin (QB7), CD45RA allophycocyanin (H100), CD56 PE-Cy7 (NCAM16.2) and (B159), CD8 PE-Cy7 and PerCP-Cy5.5 (SK7), CD8 BV421 and PE-Cy7 (3S25-5), CD8 BV605 and allophycocyanin–H7 (SK3), CD38 allophycocyanin (QB7), CD45RA allophycocyanin (H100), CD56 PE-Cy7 (NCAM16.2) and (B159), CD8 PE-Cy7 and PerCP-Cy5.5 (SK7), CD8 BV421 and PE-Cy7 (3S25-5), CD8 BV605 and allophycocyanin–H7 (SK3), CD38 allophycocyanin (QB7), CD45RA allophycocyanin (H100), CD56 PE-Cy7 (NCAM16.2) and (B159), CD8 PE-Cy7 and PerCP-Cy5.5 (SK7), CD8 BV421 and PE-Cy7 (3S25-5), CD8 BV605 and allophycocyanin–H7 (SK3), CD38 allophycocyanin (QB7), CD45RA allophycocyanin (H100), CD56 PE-Cy7 (NCAM16.2) and (B159), CD8 PE-Cy7 and PerCP-Cy5.5 (SK7), CD8 BV421 and PE-Cy7 (3S25-5), CD8 BV605 and allophycocyanin–H7 (SK3), CD38 allophycocyanin (QB7), CD45RA allophycocyanin (H100), CD56 PE-Cy7 (NCAM16.2) and (B159), CD8 PE-Cy7 and PerCP-Cy5.5 (SK7), CD8 BV421 and PE-Cy7 (3S25-5), CD8 BV605 and allophycocyanin–H7 (SK3), CD38 allophycocyanin (QB7), CD45RA allophycocyanin (H100), CD56 PE-Cy7 (NCAM16.2) and (B159), CD8 PE-Cy7 and PerCP-Cy5.5 (SK7), CD8 BV421 and PE-Cy7 (3S25-5), CD8 BV605 and allophycocyanin–H7 (SK3), CD38 allophycocyanin (QB7), CD45RA allophycocyanin (H100), CD56 PE-Cy7 (NCAM16.2) and (B159), CD8 PE-Cy7 and PerCP-Cy5.5 (SK7), CD8 BV421 and PE-Cy7 (3S25-5), CD8 BV605 and allophycocyanin–H7 (SK3), CD38 allophycocyanin (QB7), CD45RA allophycocyanin (H100), CD56 PE-Cy7 (NCAM16.2) and (B159), CD8 PE-Cy7 and PerCP-Cy5.5 (SK7), CD8 BV421 and PE-Cy7 (3S25-5), CD8 BV605 and allophycocyanin–H7 (SK3), CD38 allophycocyanin (QB7), CD45RA allophycocyanin (H100), CD56 PE-Cy7 (NCAM16.2) and (B159), CD8 PE-Cy7 and PerCP-Cy5.5 (SK7).
(Fluidigm). This microfluidic platform was then used to conduct quantitative PCR in mL reaction volumes. Threshold cycle, as a measurement of relative fluorescence intensity, was extracted from the Biomark real-time PCR analysis software. A panel of 96 preselected genes related to both NK cell and CD8 T cell biology was qualified as previously described, using a script provided courtesy of Mario Roe-derer (49). Subsequent data analysis was performed using JMP software (version 10). Initial analyses of the transcriptome data from the Fluidigm Biomark confirmed the quality of 74 of the 96 genes, although data on 22 genes were discarded because of lack of amplification.

ADCC assays

Measurement of ADCC was performed using the PanToxiLux assay (OncoImmunin, Gaithersburg, MD) similar to the previously described assay (50). HIV-1 Bal gp120 (catalog no. 4961; obtained through the National Institutes of Health AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) were used to coat target CEM.NKR.CECK cells. Optimal concentration used to coat target cells was determined for each gp120 through an 11-point titration starting with 20 μg/mL and 2-fold serial dilution. After coating CEM.NKR.CECK target cells with gp120 in 0.5% FBS–RPMI media, cells were labeled with TFL4 (OncoImmunin), a fluorescent target cell marker, for 15 min at 37°C and 5% CO2. Cells were then washed twice with 1X PBS and stained with the viability dye LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies) for 30 min at room temperature. After washing in 0.5% FBS–RPMI media, cells were counted as above, then resuspended to reach a final concentration of 8.0 × 10^6 cells/mL. At this point, sorted effector cell populations (NK cells, CD45RA^+CD57^+ CD8 T cells, and CD45RA^−CD57^− CD8 T cells) were washed in 0.5% FBS–RPMI media and resuspended to a final concentration of 24 × 10^6 cells/mL for an E:T ratio of 30:1. In a 96-well polypropylene plate, 25 μL of HIV-Ig (North American Biologicals, Miami, FL) at 0.5 mg/mL dilution was added to each well along with 75 μL of granzyme B substrate (OncoImmunin). After incubation for 5 min at room temperature, 25 μL of HIV-Ig (North American Biologicals, Miami, FL) at 0.5 mg/mL dilution was added to each well, and the plate was incubated for another 15 min at room temperature. The plate was then spun at 300 × g for 1 min and placed at 37°C and 5% CO2 for 1 h. Cells were washed twice with wash buffer and acquired on the LSR II (BD Biosciences) on the same day. Fluorophores were detected using a 488-nm 50-mW laser with 515/20 filters to detect granzyme B substrate, a 406-nm 100-mW laser with 525/50 filters to detect Aqua LIVE/DEAD stain, and a 640-nm 40-mW laser with 670/30 filters to detect TFL4 stain. Because of the spectral properties of the fluorescent molecules used in this panel, manual compensation of detected signals was performed to analyze the data. Data were analyzed by using FlowJo 9.7.5 (Ashland, OR).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (Version 6) for Macintosh (GraphPad Software, La Jolla, CA) or JMP software (version 10; SAS Institute, Cary, NC). Direct comparisons between two groups were performed using the nonparametric Mann–Whitney U test. Associations between groups were determined by Spearman rank correlation. To correct for multiple comparisons, the Benjamini–Hochberg false discovery rate (FDR) (51) was calculated for all observations. An FDR <0.05 was considered statistically significant. For paired observations, a paired t test was used. A p value <0.05 was considered statistically significant. Flow cytometry analysis and presentation of distributions were performed using SPICE version 5–12, downloaded from http://exon.niaid.nih.gov/spice (52). Comparison of distributions was performed using a Student’s t test and a partial permutation test as described previously (52).

Table 1. Descriptive statistics for study population

| HIV-1 Negative (n = 40) | HIV-1 Positive (n = 103) | HIV-1 Positive Initiating ART (n = 32) |
|------------------------|------------------------|------------------------|
| Age (y), median (IQR)  | 30 (25–35)             | 31 (26–36)             | 32 (29–38)             |
| Gender, no. (%)        |                       |                       |                       |
| Female                 | 20 (50)                | 65 (63)                | 14 (44)                |
| Male                   | 20 (50)                | 38 (37)                | 18 (56)                |
| Viral load (log_{10}/mL, median (IQR))a |               |                       |                       |
| CD4 count (cells/μL, median (IQR)) |               |                       |                       |

Whole blood from these participants was used to measure the expression of CD16 on CD8 T cells and characterize their activation profile. Viral load was measured by Roche Amplicor Monitor version 1.5, limit of detection 400 copies/mL. IQR, interquartile range; NA, not applicable.

Results

FcyRIIa^+ CD8 T cells expand in chronic untreated HIV-1 infection

HIV-1 negative (n = 40) and HIV-1 positive (n = 103) individuals from a cohort in Rakai, Uganda, were chosen for the investigation of FcyRIIa expression in CD8 T cells (Table I). The FcyRIIa^+ CD8 T cell population was identified as positive for CD3, TCRβ, CD8, and FcyRIIIA and negative for CD14, CD19, and CD4 (Fig. 1A, Supplemental Fig. 1). FcyRIIIA expression was detectable in T cells from healthy donors at a median (range) frequency of 3.8% (0.7–20.7%) of CD8 T cells (Fig. 1B). Interestingly, this population was nearly doubled in HIV-1–infected donors, in which a median frequency of 5.9% (1.3–37.9%) of CD8 T cells expressed FcyRIIIA (p < 0.001) (Fig. 1B). This expansion was positively associated with the overall CD8 T cell expansion in HIV-1–infected patients (p < 0.001, rho = 0.546) (Fig. 1C). HIV-1–associated expansion of FcyRIIa^+ CD8 T cells was not associated with the expression levels, measured as geometric mean fluorescence intensity (MFI), of FcyRIIIA on the surface of these cells (data not shown). There was no significant difference in FcyRIIIA expression levels (as measured by MFI) on FcyRIIa^+ CD8 T cells between HIV-1–infected and uninfected participants (data not shown). Interestingly, the FcyRIIa^+ CD8 T cells were more activated than their FcyRIIIA^− counterparts, as assessed by CD38 expression (p < 0.001) (Fig. 1D). They also expressed less of the inhibitory receptor PD-1 (p < 0.001) (Fig. 1E). The CD38 expression levels were inversely associated with CD4 counts, albeit weakly (p = 0.02, rho = −0.367), suggesting that the FcyRIIa^+ CD8 T cells become more activated as disease progresses (Fig. 1F).

To address the stability of the FcyRIIa^+ CD8 T cell pool over time, we studied a second cohort of Ugandan HIV-1–infected subjects (n = 32) located in Kampala, where longitudinal samples were available from before and after initiation of ART (Table I). These patients displayed a stable population of FcyRIIa^+ CD8 T cells over 12 mo of ART (Fig. 1G). However, of note, the activation levels of these FcyRIIa^+ CD8 T cells declined over the course of treatment, as measured by CD38 expression (p < 0.001) (Fig. 1H). These data show that HIV-1–infected Ugandans have an expanded population of activated TCRβ^+ CD8 T cells expressing FcyRIIIA and that this population is stable over 12 mo of ART.

Next, multiplexed assays and ELISA were used to quantify a suite of 20 soluble factors in plasma in relation to the size and activation level of the FcyRIIIA^+ CD8 T cells expressing CD38 as assessed by the LSR II plate. These patients displayed a stable population of FcyRIIa^+ CD8 T cells expressing CD38 as assessed by the LSR II plate. These patients displayed a stable population of FcyRIIa^+ CD8 T cells expressing CD38.
levels of the inflammatory cytokines IL-6 \((p = 0.011, \rho = 0.446, \text{FDR} = 0.040\)) and IP-10 \((p < 0.001, \rho = 0.582, \text{FDR} = 0.009\)), MCP-1 \((p = 0.016, \rho = 0.424, \text{FDR} = 0.048\)), TNF \((p = 0.008, \rho = 0.459, \text{FDR} = 0.036\)), and TNFRII \((p = 0.001, \rho = 0.556, \text{FDR} = 0.009\)) were observed (Fig. 1I, Table II). Similar correlations were observed for the MFI of CD38 on FcγRIIIA+ CD8 T cells and IP-10 \((p = 0.001, \rho = 0.547, \text{FDR} = 0.009\)), MCP-1 \((p = 0.009, \rho = 0.456, \text{FDR} = 0.032\)), TNF \((p = 0.009, \rho = 0.458, \text{FDR} = 0.032\)), and TNFRII \((p < 0.001, \rho = 0.569, \text{FDR} = 0.009\)). Thus, expansion and activation of the FcγRIIIA+ CD8 T cells is associated with plasma markers of HIV-driven systemic immune activation. In contrast, neither soluble markers of an innate antiviral response, such as IFN-α, nor the common indices of microbial translocation sCD14 and IFABP were associated with the size of the FcγRIIIA+ CD8 T cell population or the extent of their activation.

**FcγRIIIA+ CD8 T cells are late-stage effector cells and characterized by expression of Helios**

Because of the significant expansion and activation of FcγRIIIA+ CD8 T cells in HIV-1–infected individuals, we next investigated the detailed phenotype of these cells in HIV-infected subjects from the Rakai cohort. The combinatorial coexpression pattern of CCR7,
CD27, and CD45RA was significantly different between CD8 T cells positive or negative for FcγRIIIA (Fig. 2A, Supplemental Table I) (p < 0.001). Expression of CD45RA in the absence of CCR7 and CD27 was the dominant pattern among the FcγRIIIA⁺ CD8 T cells, consistent with a terminally differentiated status, whereas this phenotype was less common among CD8 T cells lacking FcγRIIIA (74% versus 18%, respectively) (p < 0.001). Next, the expression patterns of CD57, NKG2A, and NKG2D were evaluated, and the frequency of the subsets defined by these receptors were different in CD8 T cells containing CD57 expressing cells compared with those that did not (Fig. 2B) (p < 0.001). The majority of FcγRIIIA⁺ CD8 T cells expressed CD57 while maintaining NKG2D expression. In fact, all Boolean subsets containing CD57 expressing cells were higher in FcγRIIIA⁺ CD8 T cells compared with FcγRIIIA⁻ CD8 T cells (all p ≤ 0.001) (data not shown). The next panel examined CD161 and perforin, and comparison of the distribution of cell subsets expressing combinations of these two markers again revealed differences between the FcγRIIIA⁺ and FcγRIIIA⁻ CD8 T cells (Fig. 2C) (p < 0.001). The vast majority of FcγRIIIA⁺ CD8 T cells expressed perforin as compared with ~20% of FcγRIIIA⁻ CD8 T cells. In summary, FcγRIIIA⁺ CD8 T cells are distinct from their FcγRIIIA⁻ CD8 T cell counterparts by lack of CD27 expression, higher proportion of cells expressing CD57, and their predominantly perforin positivity.

The patterns of expression of maturation markers observed in FcγRIIIA⁺ CD8 T cells in HIV-1–infected donors were not significantly different from HIV-1–uninfected control subjects (all p values >0.05) (data not shown), suggesting that the elevated levels of FcγRIIIA⁺ CD8 T cells in infected individuals represent an expansion of a phenotypic cell subset retaining relatively normal characteristics. To address this question further, we investigated the expression of killer Ig-like receptors (KIRs) in CD8 T cells and NK cells expressing FcγRIIIA, as well as in late-stage differentiated CD8 T cells defined by coexpression of CD45RA and CD57 and memory CD8 T cells negative for these markers (Fig. 2D). In uninfected donors, T cell populations lacking FcγRIIIA had low levels of KIR expression, whereas NK cells had high KIR levels in diverse combinations. The FcγRIIIA⁺ CD8 T cells displayed a pattern intermediate between T cells and NK cells. Strikingly, this pattern was altered in HIV-1–infected subjects whose FcγRIIIA⁺ CD8 T cells had adopted a KIR coexpression profile very similar to that of NK cells (p < 0.001 for FcγRIIIA⁺ CD8 T cells in HIV-1–uninfected donors compared with HIV-1–infected donors; p = 0.250 for FcγRIIIA⁺ CD8 T cells compared with NK cells in HIV-1–infected donors).

T cell differentiation and maturation are controlled by a set of transcription factors, including T-bet, Eomes, and Helios. PBMC from HIV-infected donors were stained intracellularly for these transcription factors, and their expression patterns were analyzed in CD8 T cells lacking or expressing FcγRIIIA, as well as in NK cells (Fig. 3A). FcγRIIIA⁺ CD8 T cells displayed a T-bet, Eomes, and Helios expression pattern distinct from both the general CD8 T cell population and from CD56dim NK cells, with higher levels of coexpression as compared with FcγRIIIA⁺ CD8 T cells. Coexpression of all three transcription factors was common in FcγRIIIA⁺ CD8 T cells and also relatively frequent in NK cells but uncommon in the general CD8 T cell pool. Notably, 61% of the FcγRIIIA⁺ CD8 T cells expressed Helios, and this was significantly higher compared with the FcγRIIIA⁺ CD8 T cells and NK cells (p < 0.001), in which a median of 10 and 28% expressed Helios, respectively. Characterization of T-bet and Eomes can be discriminated, based on a continuum of expression and varies on lymphocyte subsets (53). FcγRIIIA⁺ CD8 T cells were dominated by a high T-bet expression profile with variable Eomes expression that was very similar to CD16⁺ NK cells (Fig. 3B, 3C). HIV-1 infection status had minimal effect on T-bet and Eomes in these populations. FcγRIIIA⁺ CD8 T cells showed a much more variable expression pattern of both transcription factors, which may reflect the different states of maturation and differentiation within this compartment.

Altogether, these data indicate that the FcγRIIIA⁺ CD8 T cell population expanded in HIV-1–infected people is characterized by Helios expression and has a late-stage differentiated effector phenotype. This population mostly retains the characteristics seen in healthy donors as it expands during HIV-1 infection, although KIR expression is significantly elevated.

The FcγRIIIA⁺ CD8 T cell transcriptome reveals a mixed effector CD8 T cell and NK cell character

To better understand the identity of the FcγRIIIA⁺ CD8 T cells, we next analyzed their transcriptional profile by Fluidigm Biomark. A
Panel of 96 genes involved in T cell function or NK cell function was selected (Supplemental Table II), and the expression of these genes was analyzed in cell populations purified by flow cytometry sorting. For these analyses, cells from seven HIV-1–infected donors were sorted into four populations, 500–1000 cells per population: 1) CD45RA+CD57+ CD8 T cells expressing FcγRIIIA, 2) CD45RA+CD57+ CD8 T cells lacking expression of FcγRIIIA, 3) CD45RA-CD57- CD8 T cells not expressing FcγRIIIA, and 4) CD56dimCD16+ NK cells. The data for 74 out of the 96 genes passed quality control, and principal component

**FIGURE 2.** FcγRIIIA+ CD8 T cells display a late-stage effector phenotype in chronic untreated infection. A detailed phenotype of FcγRIIIA+ CD8 T cells after gating on small lymphocytes, singlets, Aqua LIVE/DEAD+, CD8+CD3+ T cells in HIV+ (n = 15) and HIV− (n = 15) individuals was examined. (A) Expression of CD27, CCR7, and CD45RA in CD8 T cell subsets having or lacking FcγRIIIA surface expression. (B) Expression of CD57, NKG2A, and NKG2D in CD8 T cell subsets having or lacking FcγRIIIA surface expression. (C) Expression of CD161 and perforin in CD8 T cell subsets having or lacking FcγRIIIA surface expression. (D) Analysis of KIR surface expression patterns in CD45RA+CD57+CD8 T cells, CD45RA+CD57−CD8 T cells, CD56dim NK cells, and CD45RA−CD57− CD8 T cells.
analysis (PCA) was performed on the total data set of expression of these 74 genes in all four of the cell subsets (Fig. 4A). Notably, the transcriptional profile of FcγRIIIA+ CD8 T cells overlapped with both the CD45RA⁻CD57⁻ CD8 T cells lacking expression of FcγRIIIA and the CD56dimCD16⁺ NK cells, whereas the CD45RA⁺CD57⁺ memory CD8 T cell subset was
most distant. Principal component 1 contributed 26% of the variability in the data set, and component 2 contributed 14.7%.

Expression of genes GZMB, LAIR1, GZMK, PRF1, and CD244 contributed most to principal component 1, and genes GZMK, IL-6ST, TGFB1, CD38, and CD160 contributed most to principal component 2.

A subset of genes showed expression patterns that segregated the FcyRIIIA+ CD8 T cell population from the NK cells and the FcyRIIIA2 CD8 T cell populations (Fig. 4B, Supplemental Fig. 2). Notably, the FcyRIIIA+ CD8 T cell displayed significantly higher IKZF2 expression than any of the three other reference populations and lower IL-7R expression than the other T cell populations and at levels similar to CD56dimCD16+ NK cells. Regarding a range of genes encoding NK cell–associated receptors, including KIR2DS2; KIR2DS1; KIR3DL1; KIR3DS1; KLR2C; KLR3; KLR1; KLR1; KLR4-1, and NCR1, the FcyRIIIA+ CD8 T cells showed a pattern intermediate between FcyRIIIA+ CD45RA+CD57+CD8 T cells and the CD56dimCD16+ NK cells. In fact, KLRF1 encoding the NK cell–associated receptor NKp80, expressed at the highest levels by CD56dimCD16+ NK cells, was expressed at significantly higher levels when compared with the FcyRIIIA+ terminal effector CD8 T cells and effector memory CD8 T cells. Compared with their FcyRIIIA2 counterparts, the FcyRIIIA+ CD8 T cells also expressed higher levels of genes involved in regulating T cell function, including TNFSF13B. Additionally, the FcyRIIIA+ CD8 T cells had significantly lower expression of TGFBR1 than the CD56dim NK cells, but levels were above that of the other CD8 T cell populations. Altogether, the gene expression analysis indicates that FcyRIIIA+ CD8 T cells have a transcriptional profile intermediate between effector CD8 T cells and CD56dim NK cells.

Because of the distinct transcriptional signature of FcyRIIIA+ CD8 T cells, we were interested in confirming expression of the IL-7R and...
KLRF1 genes at the protein level. We further examined 10 chronically HIV-1–infected and 10 uninfected individuals for surface expression of these receptors by flow cytometry. The majority of FcγRIIIA+CD45RA+CD57+CD8 T cells expressed NKp80 (median 68%) and lacked expression of the IL-7 receptor, CD127 (median 2%) (Fig. 4C). No differences were observed in FcγRIIIA+CD45RA+CD57+CD8 T cells expressing NKp80 or IL-7Rα between HIV-1 positive and negative individuals, and no relationship was observed between expression and markers of HIV-1 disease progression. IL-7Rα protein expression was similar between NK cells and CD45RA+CD57+CD8 T cells, irrespective of FcγRIIIA+ expression. Interestingly, NKp80 was only found at appreciable levels in the T cells with the FcγRIIIA+CD45RA+CD57+ phenotype. Together, the FcγRIIIA+CD8 T cells have a distinct NKp80+IL-7Rα− character different from other effector CD8 T cells and more akin to CD56dim NK cells.

**Potent HIV-specific ADCC activity mediated by FcγRIIIA+CD8 T cells**

ADCC is part of the repertoire of effector functions employed by NK cells to detect and target HIV-1–infected cells. Recent data indicating that nonneutralizing Ab-mediated effects may contribute to HIV vaccine efficacy have spurned a renewed interest in ADCC as a protective mechanism (54, 55). The present observation that HIV-1 infection drives the expansion of late-stage effector CD8 T cells with a hybrid NK cell–CD8 T cell character, including FcγRIIIA and lytic protein expression, suggests that CD8 T cells might actually mediate ADCC. To test this possibility, effector cell populations from HIV-1–infected individuals were sorted by flow cytometry, and the ability of these cells to mediate ADCC against HIV BaL gp120-coated CEM.NKRI+CCR5 target cells was evaluated by the PanToxiLux granzyme B substrate cytotoxicity assay (Fig. 5A). To avoid FcγRIIIA downregulation or blocking because of staining, CD45RA+CD57+CD8 T cells were sorted to enrich for FcγRIIIA+ cells (9–21% FcγRIIIA+) and then compared with FcγRIIIA−CD45RA+CD57− memory CD8 T cells and with NK cells sorted from the same donors. In the presence of HIV-Ig, the CD45RA+CD57+ cells from three HIV+ donors clearly mediated ADCC, as did the NK cells, whereas the CD45RA−CD57−CD8 T cell population did not (Fig. 5A, 5B). As such, bulk CD45RA+CD57+CD8 T cells performed ADCC.

**FIGURE 5.** HIV-specific ADCC mediated by FcγRIIIA+ CD8 T cells. (A) Representative FACS plots of the cytolysis PanToxiLux assay from HIV+ (n = 3) individuals. (B) HIV-1 gp120-specific ADCC mediated by HIV-Ig. (C) Comparison of ADCC mediated by FcγRIIIA+CD8 T cells and NK cells on a per FcγRIIIA+ cell basis.
lower than the NK cells (Fig. 5B). However, after adjusting for the frequency of FcγRIIIA expression in these populations, 9–21% in CD45RA+CD57+ CD8 T cells and 69–96% in CD56dim NK cells, ADCC capacity of FcγRIIIA+ CD8 T cells was similar to that of FcγRIIIA+ NK cells (Fig. 5C). Interestingly, the FcγRIIIA MFI on FcγRIIIA+ CD8 T cells was significantly lower compared with FcγRIIIA+ MFI on CD56dim NK cells ($p < 0.001$). FcγRIIIA+ CD8 T cell ability to mediate ADCC, based on normalized FcγRIIIA MFI or the integrated MFI (frequency multiplied by the MFI), was as good as NK cells (data not shown). These data demonstrate that the FcγRIIIA+ CD8 T cell population expanding during chronic HIV-1 infection can mediate HIV-specific ADCC at levels comparable to NK cells.

**Discussion**

CD8 T cells use a range of effector functions to combat viral infections, including cytolyis and effects mediated by cytokines and chemokines. A hallmark of these antiviral functions is that they depend on the exquisite Ag specificity of TCRs and their recognition of viral Ag in an MHC-restricted manner. In this study, we demonstrate that late-stage effector CD8 T cells acquire FcγRIIIA expression in HIV-1–infected individuals and use this Fc receptor to mediate HIV-specific ADCC in the absence of TCR recognition of Ag. Using a commercial in vitro assay, commonly used in assessing HIV-1 ADCC activity (50), we measured the effector capacity, on a per cell basis, of FcγRIIIA+ CD8 T cells to mediate Ag-specific ADCC against gp120-coated targets as efficiently as NK cells from the same donors. These findings indicate that in the context of chronic uncontrolled HIV-1 infection, a significant subset of CD8 T cells acquires innate characteristics and performs a function in the immune system normally associated with NK cells. Functional diversification of adaptive CD8 T cells may be important as therapeutic strategies evolve to include Ab-mediated mechanisms to eliminate HIV-1 reservoirs (56–58).

In the Ugandan population studied in this work, expression of FcγRIIIA occurs on ~5% of CD8 T cells from healthy donors, and this frequency is doubled in patients with chronic untreated HIV-1 infection. In fact, some patients have more than 30% of their CD8 T cells expressing FcγRIIIA. The finding that the size of this population is positively associated with the global CD8 T cell expansion in these patients suggests that the FcγRIIIA+ CD8 T cells expand in response to the chronic uncontrolled viral replication. These expanded cell populations have a terminally differentiated phenotype with frequent expression of CD45RA, CD57, and perforin but little expression of CD27 and CCR7, further supporting this notion. The phenotypic profile of these cells is similar between HIV-1–infected patients and healthy donors [data not shown and (23)]. However, we found one exception to this observation; the FcγRIIIA+ CD8 T cells adopt a KIR expression profile similar to NK cells in HIV-1–infected subjects, an observation not seen in healthy donors (Fig. 2D). The conditions in vivo during HIV-1 infection thus seem to drive not only an expansion of these cells but also expression of surface receptors beyond FcγRIIIA normally associated with NK cells and reflective of the rise in terminally differentiated CD8 T cells in chronic viral infections (59).

These functional and, to some extent phenotypic, similarities with NK cells led us to ask how the FcγRIIIA+ CD8 T cells relate to FcγRIIIA+ T cell subsets as well as FcγRIIIA+ NK cells on the transcriptional level. Based on a supervised transcriptional analysis of 74 genes in seven donors, the FcγRIIIA+ CD8 T cells appear to have a transcriptional program intermediate between late-stage effector CD8 T cells lacking FcγRIIIA and CD56dim NK cell expressing FcγRIIIA. Most interestingly, transcript and protein levels for KLRF1, encoding the activating NKp80 receptor, were expressed at high levels similar to NK cells compared with effector memory or FcγRIIIA+ CD8 T cells. NKp80 has recently been shown to associate with the development and maturation of fully functional NK cells (60). Whereas FcγRIIIA+ CD8 T cells show some features similar to CD56dim NK cells, PCA revealed that FcγRIIIA+ CD8 T cells, FcγRIIIA+ CD8 T cells, and CD56dim NK cells were distinct from the CD45RA+CD57− memory CD8 T cell population. Consistent with this notion, when the genes differentially expressed between the FcγRIIIA+ CD8 T cell and the effector memory T cell population were entered into the Reactome pathway analysis database, the DAP12 pathway, implicated in activation of NK cells, was indicated as enriched in the FcγRIIIA+ CD8 T cells (Supplemental Fig. 2, Supplemental Table II) (61, 62). Furthermore, we observed the upregulation of 10 genes in FcγRIIIA+ CD8 T cells compared with the effector memory CD8 T cell population that are associated with NK-like rapid effector function and the “innateness gradient” defined by Gutierrez-Arcelus et al. (63) including GZMB, PRF1, KIR3DL1, KLRK1, KLRD1, KLRF1, NCR1, KLRK1, KLRD2, and ITGAM (Supplemental Fig. 2, Supplemental Table II). Although the overall pattern is that FcγRIIIA+ CD8 T cells overlap with both FcγRIIIA+ T cells and CD56dim NK cells, these cells also manifest distinctive features somewhere between innate and adaptive immune cells (64). A pattern that stands out is the high expression by FcγRIIIA+ CD8 T cells of the transcription factor Helios, encoded by the IKZF2 gene, both at the protein and gene levels. These cells also have very low expression of IL-7Rα. The low IL-7Rα expression level is consistent with a model in which these cells are either maintained by non–IL-7–dependent factors or, rather, short-lived in vivo. Our finding that patients initiating ART largely maintain the expanded FcγRIIIA+ CD8 T cell population over 12 months suggests that these cells are not intrinsically short-lived, and thus may even be maintained by IL-7–independent mechanisms. This interpretation is supported by the recent finding of expansion of long-lived effector CD45RA+ CD8 T cells that are IL-7Rαlow KLRG1high in latent CMV and EBV infection, a population which phenotypically overlaps with the FcγRIIIA+ CD8 T cell identified in this study (65).

The expansion of FcγRIIIA+ CD8 T cells we observe in this study is reminiscent of the expansion of CD8 T cells with a similar phenotype in hepatitis C virus (HCV)–infected patients (23). Whereas HIV-1 and HCV differ in target cell tropism and mechanisms of pathogenesis, for example, they have in common establishment of chronic infections that are very difficult for the immune system to control. This is partly because of the shared features of rapid viral replication and high mutation rates. These features lead to selection of epitope immune escape variants that allow these viruses to avoid efficient recognition by clonally expanded populations of T cells. Viral quasispecies mutate away from the originally transmitted viral sequence under T cell selection pressure and some of the early responding epitope-specific T cell populations may thus lose their efficiency in targeting infected cells. Future studies are warranted to test the hypothesis that accumulation of FcγRIIIA+ CD8 T cells may be a clonally driven process and this could be addressed by TCR repertoire analysis. The FcγRIIIA+ CD8 T cells have a phenotype that would be expected from a T cell population expanded by Ag recognition, because they are largely negative for CD27 and CCR7, but positive for CD57, perforin and CD45RA. In the yellow fever virus vaccine model, the yellow fever vaccine–specific CD8 T cells are CD45RO+ during the peak of the effector response and then revert back to CD45RA expression as the Ag is cleared and memory is established (66, 67). This is consistent with a model in which
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