Single-Cell Level Response of HIV-Specific and Cytomegalovirus-Specific CD4 T Cells Correlate With Viral Control in Chronic HIV-1 Subtype A Infection

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Background and Objective: HIV-1 subtype A is the second most prevalent subtype globally and is associated with reduced viral load, higher CD4 absolute counts, and slower disease progression. To study the possible role of T cells associated with better outcome, we examined CD4 and CD8 T-cell responses against HIV-1 and cytomegalovirus (CMV) in Ugandans infected with subtype A HIV-1.

Methods: T-cell responses were investigated using flow cytometry and novel subtype A variant inclusive peptide (VIP) sets designed for this evaluation. CD4 T-cell responses focused primarily on Gag, whereas CD8 T-cell responses were broadly directed against Gag, gp41, and Nef VIP sets. CD4 T cells primarily responded with interferon (IFN)-γ, whereas CD8 cells were more diverse with degranulation (CD107a), IFN-γ, and macrophage inflammatory protein (MIP)-1β production.

Results: No relationship was observed between CD8 T-cell responses and the HIV-1 load. Similarly, the frequency of CD4 T cells responding to these antigens did not associate with viral control. However, in CD4 T cells responding against Gag or CMV, the IFN-γ intensity, indicative of the production at the single-cell level, was inversely proportional to viral load. No significant relationship was found between T-cell effector/memory phenotype and viral control.

Conclusions: The per cell production of IFN-γ in CD4 T cells responding to HIV-1 or CMV correlated with viral control in chronic HIV-1 subtype A infection. These data suggest that quantitative aspects at the single-cell level may be more important than the frequency of antigen-specific CD4 T cells in HIV-1 subtype A infection control.

Key Words: T cells, HIV-1, AIDS, viral infections, memory

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INTRODUCTION

The largest burden of the HIV-1 epidemic is in sub-Saharan Africa where two thirds of the global infections are found.[1] HIV-1 subtype A represents a growing epidemic that accounts for more than 12% of infections globally and is predominantly observed in Africa but increasing in Asia and Europe.[2] In Uganda, more than 1.2 million people are infected with HIV-1, contributing to a prevalence of more than 6%,[1] with HIV-1 subtypes A and D most prevalent and a growing number of recombinant forms emerging.[3–5] HIV-1 subtype A has been reported to progress more slowly to AIDS and death as compared with other subtypes or recombinant strains in cohorts from Kenya and Tanzania,[6] Senegal,[7] and Uganda.[8,9] In addition, evidence suggests that due to the higher transmissibility and slower disease progression, HIV-1 subtype A represents a higher proportion of infections currently found in Uganda compared with the previous decade.[10] Together, these data suggest that HIV-1 subtype A–infected cohorts represent a relevant and novel population to examine the potential role of cellular immune responses in a less progressive infection.

In primary HIV-1 infection, the appearance of HIV-1–specific CD8 T cells temporally correspond to a decline in peak viremia.[11–14] Further evidence of CD8 T-cell control of HIV-1 comes from nonhuman primate models where depletion of CD8 lymphocytes results in elevated viral loads and prolonged CD4 T-cell loss.[15–17] Additionally, in humans, discrete CD8 T-cell functional profiles are associated with slower disease progression,[18] and patients who exhibit responses directed toward certain viral proteins have more favorable clinical outcomes.[19,20] Helper CD4 T cells are also

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believed to play a critical role in the control of HIV-1 viremia because CD4 T-cell proliferation to p24 antigen is inversely associated with viremia, independently of CD4 T-cell counts.[21,22] Certain functional profiles including interleukin (IL)-2 production, with or without interferon (IFN)-γ, have been associated with lower HIV-1 viremia.[23] Furthermore, cytotoxic activity is a feature of some HIV-specific CD4 T cells.[24] Despite the numerous descriptions and associations of T cells in HIV-1 infection, the exact profile of a protective T-cell response is not clearly understood.

Characterization of distinct subsets of T cells based on CD45 isoforms and homing markers has generated a number of hypotheses about maturation and differentiation.[25–28] In HIV-1 infection, a distinct maturational phenotype is observed with increased preterminally differentiated HIV-specific T cells. This may be due to persistent antigen exposure.[29] Moreover, certain T-cell phenotypes can be associated with increased functionality and are more frequent in HIV-1 long-term nonprogressors compared with progressors.[1,18] Given these observations, we examined the aspects of T-cell function and phenotype in Ugandans with HIV-1 subtype A chronic infection using specially designed variant inclusive peptide (VIP) sets to Gag, gp41, and Nef from known subtype A sequences.

METHODS

Volunteers, Samples, and Diagnostic Testing

Study participants aged 19–48 years were enrolled in a prospective community-based cohort to assess the prevalence and incidence of HIV-1 infection in Kayunga district, Uganda.[30] Blood was collected at baseline, and 6 months and 12 months after enrollment. Peripheral blood mononuclear cells (PBMC) were obtained from acid-citrate-dextrose anticoagulated whole blood within 6 hours of venipuncture, as previously described.[31] PBMC were cryopreserved in media containing 10% dimethyl sulfoxide and stored in the vapor phase of liquid nitrogen. Autologous plasma and serum were stored at −80°C for diagnostic testing. Institutional Review Boards of United States and Uganda approved this study, and all volunteers provided written informed consent. HIV-1 testing and serology for hepatitis B virus, hepatitis C virus, syphilis, and herpes simplex virus 2 were performed, as previously described.[32]

HIV-1 Subtype A VIP Set Design

Subtype A VIP sets to Gag, gp41, and Nef, as well as the cytomegalovirus (CMV) pp65 peptide pool used for stimulation were synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). The Gag VIP set consisted of a total of 207 peptides with lengths varying from 15 to 18 amino acids (median of 16) overlapped by 10 to 12 amino acids (see **Figure 1A, Supplemental Digital Content 1**, http://links.lww.com/QAI/A333). The gp41 VIP set consisted of a total of 113 peptides with lengths varying from 15 to 18 amino acids (median of 16) and overlapped by 10 to 12 amino acids (see **Figure 1B, Supplemental Digital Content 1**, http://links.lww.com/QAI/A333). The Nef VIP set consisted of a total of 85 peptides with lengths varying from 15 to 18 amino acids (median of 16) and overlapped by 10 to 12 amino acids (see **Figure 1C, Supplemental Digital Content 1**, http://links.lww.com/QAI/A333). Peptides containing common amino acid variants were included to maximize the coverage potential for subtype A viruses based on the Los Alamos National Lab HIV Sequence Database 2008 alignment data set. The 10-mer T-cell epitope coverage for exact matching provided by the A-Gag VIP, A-gp41 VIP, and A-Nef VIP peptide sets for subtype A was calculated at 72.1%, 63.6%, and 62.7%, respectively. The human CMV peptides were commercially designed and available from JPT Peptide Technologies GmbH. The single-peptide pool consisted of 138 peptides, 15 amino acids in length, overlapped by 11 amino acids across the entire CMV pp65 protein.

Intracellular Cytokine Staining and Polychromatic Flow Cytometry

Two polychromatic flow cytometry based assays were used to measure T-cell phenotype and function. A 5-function assay was performed from PBMC stimulated for 6 hours with 1 μg/mL of HIV-1 subtype A VIP Gag, VIP gp41, VIP Nef, CMVpp65 pool, and staphylococcal enterotoxin B or in the absence of stimulation. PBMC were thawed in 10% fetal bovine serum containing media supplemented with Benzonase nuclease, and counts and viabilities were performed using Guava ViaCount reagent and the Guava PCA (Guava Technologies, Hayward, CA). Cells were then stimulated in the presence of monoclonal antibodies against CD28/CD49d, and the CD107a marker of degranulation in 96-well polypropylene U-bottom plates at 5%CO_2 at 37°C with 90% relative humidity for 6 hours. After 2 hours of stimulation, brefeldin A and monensin were added to inhibit protein transport. One million cells were used per stimulation condition. Cells were washed and stained with Aqua Live/Dead stain (Molecular Probes, Eugene, OR). Again, cells were washed, blocked using normal mouse IgG (Caltag, Eugene, OR), and surface stained for CD14 and CD19 Alexa 700. Cells were washed, fixed in 2% formaldehyde for 15 minutes, and washed again. Cells were then permeabilized using Perm/Wash (Becton Dickinson, San Jose, CA) and stained intracellularly with CD8 PerCP-Cy5.5, IFN-γ eFluor450, IL-2 allophycocyanin (APC), CD3 APC-H7, MIP-1β phycoerythrin (PE), CD4 energy coupled dye, and tumor necrosis factor (TNF)-α PE-Cy7. Cells were washed and run on a FACS LSRII (BD Biosciences).

The second polychromatic panel was developed to measure the phenotype of HIV-specific T cells stimulated with HIV-1 subtype A Gag and staphylococcal enterotoxin B or in the absence of stimulation. Samples were stained, using a similar procedure as above, with an 11-color panel to assess the phenotype (Aqua Live/Dead, CD3 APC-H7, CD4 energy coupled dye, CD8 Qdot605, CD14 Alexa700, and CD19 Alexa700), memory status (CD45RO FITC, CD62L PE-Cy7, and CCR7 APC), and function (IFN-γ Pacific Blue, IL-2 PerCP-Cy5.5, and TNF-α PE) of T cells. TNF-α cleaving enzyme inhibitor was used to prevent the loss of CD62L, as previously reported.[33] All samples were acquired on a FACS

LSRII and analyzed using FlowJo version 9.3.1. A positive functional response was defined as ≥0.05% gated positive cells and at a frequency of ≥3 times the unstimulated control sample, as previously used in our HIV-1 vaccine studies.[34] Sample gating strategy used to characterize T-cell function is outlined in **Supplemental Digital Content 2** (see **Figure 2**, http://links.lww.com/QAI/A333) and maturational definitions are outlined in **Supplemental Digital Content 3** (see **Figure 3**, http://links.lww.com/QAI/A333).

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism Software version 5.0a for Mac OSX (GraphPad Software, La Jolla, CA). Comparisons between the groups were performed using the nonparametric Mann–Whitney *U* test for continuous data. Associations between outcomes were determined by Spearman rank correlation. P values < 0.05 were considered statistically significant. Analysis and presentation of distributions was performed using SPICE version 5-1.2 (downloaded from http://exon.niaid.nih.gov/spice).[35] Comparison of distributions was performed using a Student *t* test and a partial permutation test as described.[35]

RESULTS

Ugandans Infected With HIV-1 Subtype A Respond to Subtype-Optimized VIP Sets

Study participants were selected from a cohort to assess the prevalence and incidence of HIV-1 infection in Kayunga district, Uganda.[30] Forty-eight chronically infected, treatment-naive individuals were selected based on HIV-1 subtype and sample availability (Table 1). HIV-1–infected participants displayed a wide range of HIV-1 viral load and CD4 T-cell count. Prevalence of hepatitis B, C, and syphilis was relatively low in the study cohort (all <10% at screening), whereas herpes simplex virus 2 was much more common with 90% of the individuals reactive in the screening enzyme-linked immunosorbent assay. Neutropenia and eosinophilia were common in this population, as previously reported for this region of Africa.[36–39]

To study HIV-1–specific T-cell immune responses, peptide sets were specifically designed to cover subtype A HIV-1 sequence diversity, so that a comprehensive coverage of HIV-1–specific responses could be obtained. Peptide sets were designed using an N-mer (10 amino acid window) identity algorithm to cover the majority of sequence diversity at the T-cell epitope level for the HIV-1 subtype A Gag, gp41, and Nef proteins. Peptides containing common amino acid variants have been included to maximize the coverage potential for subtype A viruses. For example, the HIV-1 subtype A Gag VIP set provides a mean coverage of 73% (range: 10%–100%) of all reported 10 amino acid sequences found in the Los Alamos National Lab database for subtype A Gag (146 sequences) (see **Figure 1A, Supplemental Digital Content 1**, http://links.lww.com/QAI/A333). Similar coverage was observed for HIV-1 subtype A gp41 and Nef peptide pools (see **Figure 1B and 1C**, respectively, **Supplemental Digital Content 1**, http://links.lww.com/QAI/A333).

TABLE 1. Study Population Descriptive Statistics

Subjects, n	48
Sex, n (%)	
Female	30 (63)
Male	18 (38)
Median age (range), yr	31 (19–48)
Immune phenotype	
CD4 Absolute, median (range), cells/mL	449 (3–1350)
CD4, median percent (range)	26 (1–44)
CD8 Absolute, median (range), cells/mL	682 (108–2726)
CD8, median percent (range)	41 (21–68)
NK cell Absolute, median (range), cells/mL	209 (43–994)
NK cell, median percent (range)	12 (4–40)
B-cell absolute, median (range), cells/mL	191 (20–504)
B cell, median percent (range)	11 (3–41)
Neutrophil, median percent (range)	38 (12–72)
Eosinophil, median percent (range)	11 (1–45)
Viral load, median (range), copies/mL	50,011 (2702–>750,000)
HIV-1 subtype	
HIV Clade A, n (%)	48(100)
Serum albumin (g/dL)	3.8 (2.0–4.8)
Hepatitis B, n (%)	2 (4)
Hepatitis C, n (%)	1(2)
Syphilis, n (%)	5(10)
Herpes simplex virus 2, n (%)	43 (90)

PBMC from 48 HIV-1 chronically infected, treatment-naive Ugandans were tested with a polychromatic multifunctional assay to examine degranulation (CD107a), cytokine production (IL-2, IFN-γ, and TNF-α), and chemokine production (MIP-1β) in T cells responding to stimulation with the HIV-1 subtype A VIP Gag, gp41, Nef pools, or CMVpp65. Overall, CD8 T-cell responses were readily detectable against all 3 HIV-1 proteins and CMVpp65, whereas CD4 responses were mainly observed against the Gag VIP set and CMVpp65 (Figs. 1A, B). HIV-1–specific responses displayed a dynamic magnitude range, and CD4 T-cell responses were strongest against the Gag VIP set (Fig. 1C). The CD8 T-cell responses were far more substantial for all 3 HIV-1 proteins and predominantly consisted of degranulation (CD107a), IFN-γ, and MIP-1β production (Fig. 1D). Similarly, CMV-specific responses displayed dynamic magnitude range for both CD4 T cells (Fig. 1C) and CD8 T cells (Fig. 1D).

Antiviral CD4 T-Cell IFN-γ Geometric Mean Fluorescence Intensity Is Inversely Proportional to HIV-1 Load

Because the CD4 T-cell population displayed substantial HIV-1–specific response, the quality and magnitude was assessed and correlated with HIV-1 viral load to further explore the possible relationships between CD4 T-cell function and control of virus. Strikingly, the geometric mean fluorescence intensity of IFN-γ production to the HIV-1 subtype A VIP Gag set in CD4 T cells was inversely proportional to

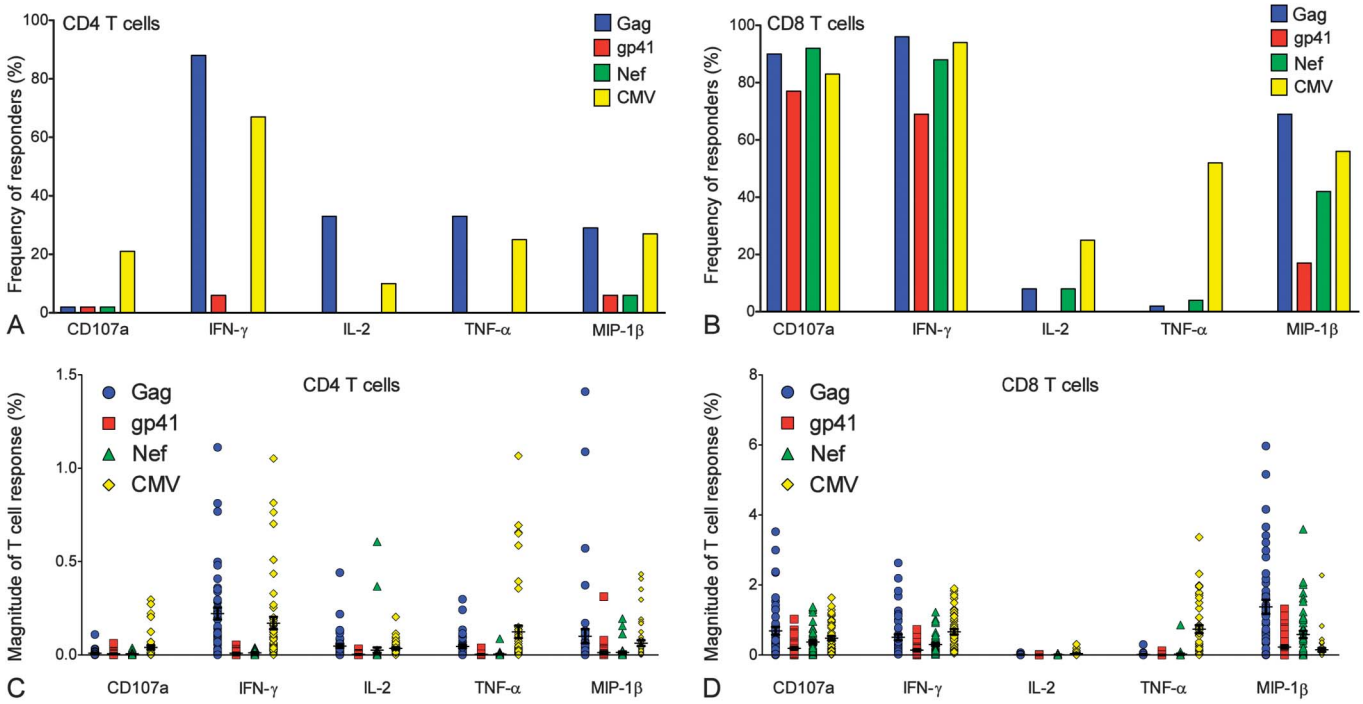

FIGURE 1. HIV-1–specific T-cell responses to subtype A VIP sets. PBMC from 48 HIV-1 subtype A, chronically infected, and treatment-naive individuals were thawed and assessed in a standard 5-function polychromatic flow cytometry assay. PBMC were stimulated with peptides or in the absence of stimulation, and HIV-1–specific responses were corrected by subtracting the unstimulated control response from the test peptide response. A, Bar chart showing the percentage of patients displaying a detectable CD4 T cell response to Gag (blue), gp41 (red), and Nef (green) VIP sets, as well as the CMVpp65 peptide pool (yellow). B, Bar chart showing the percentage of patients displaying a detectable CD8 T-cell response to Gag (blue), gp41 (red), and Nef (green) VIP sets, as well as the CMVpp65 peptide pool (yellow). C, Scatter plots showing the percentage of the adjusted CD4 T-cell response with the mean and standard error of the mean bars plotted in black. Individual functions are denoted on the *x*-axis and specific responses are coded as follows: Gag (blue circles), gp41 (red squares), Nef (green triangles), and CMV (yellow diamonds). D, Scatter plots showing the percentage of the adjusted CD8 T-cell response with the mean and standard error of the mean bars plotted in black. Individual functions are denoted on the *x*-axis and responses are coded as follows: Gag (blue circles), gp41 (red squares), Nef (green triangles), and CMV (yellow diamonds).

HIV-1 viral load ($\rho = -0.441$; $P = 0.002$) (Fig. 2A). The frequency of circulating CD4 T cells responsive with IFN-γ production to the VIP Gag set showed no relationship to HIV-1 viral load ($\rho = -0.041$; $P = 0.779$) (Fig. 2B).

HIV-1–specific CD8 T-cell responses have been associated with viral control and disease progression in HIV-1 subtype B infection, but little is known with regard to HIV-1 subtype A infection.[18] The quality and magnitude of the HIV-1–specific CD8 response were compared with markers of disease progression, HIV-1 viral load, and absolute CD4 counts. The geometric mean fluorescence intensity of IFN-γ production to the Gag VIP set in CD8 T cells was not associated with viral load ($\rho = -0.213$; $P = 0.142$) (Fig. 2C). Similarly, the frequency of CD8 T cells responding with IFN-γ production to the subtype A Gag VIP set showed no significant relationship to HIV-1 viral load ($\rho = -0.236$; $P = 0.107$) (Fig. 2D).

To evaluate T-cell responses of another specificity in this context, we analyzed the CD4 and CD8 T-cell responses against CMVpp65 in the same subtype A–infected patients. Similar to the CD4 T-cell response against HIV-1 Gag, the geometric mean fluorescence intensity of IFN-γ in CMV-specific CD4 T cells was inversely proportional to HIV-1 viral load ($\rho = -0.357$; $P = 0.013$) (Fig. 2E). In contrast, the frequency of CMV-specific CD4 and CD8 T cells or the geometric mean fluorescence intensity of IFN-γ in CMV-specific CD8 T cells showed no significant relationship to HIV-1 viral load (Figs. 2F–H). These data indicate that the quantity of IFN-γ produced at the single-cell level by both HIV-specific and CMV-specific CD4 T cells is associated with viral control in HIV-1 subtype A infection in Uganda.

HIV-1 Subtype A–Specific T Cells Display Effector and Effector Memory Phenotypes

PBMC from the 48 HIV-1 subtype A–infected Ugandans were further examined for maturation (CD45RO, CCR7, and CD62L) and function (IL-2, IFN- γ, and TNF-α) of CD4 and CD8 T cells responding to stimulation with the HIV-1 subtype A Gag VIP set. CD4 and CD8 T-cell memory definitions and trends are shown in **Supplemental Digital Content 2** (see **Figure 2**, http://links.lww.com/QAI/A333). HIV-1 Gag-specific CD4 T-cell responses were primarily of an effector memory (T_{EM}) phenotype with a median 90% (range:

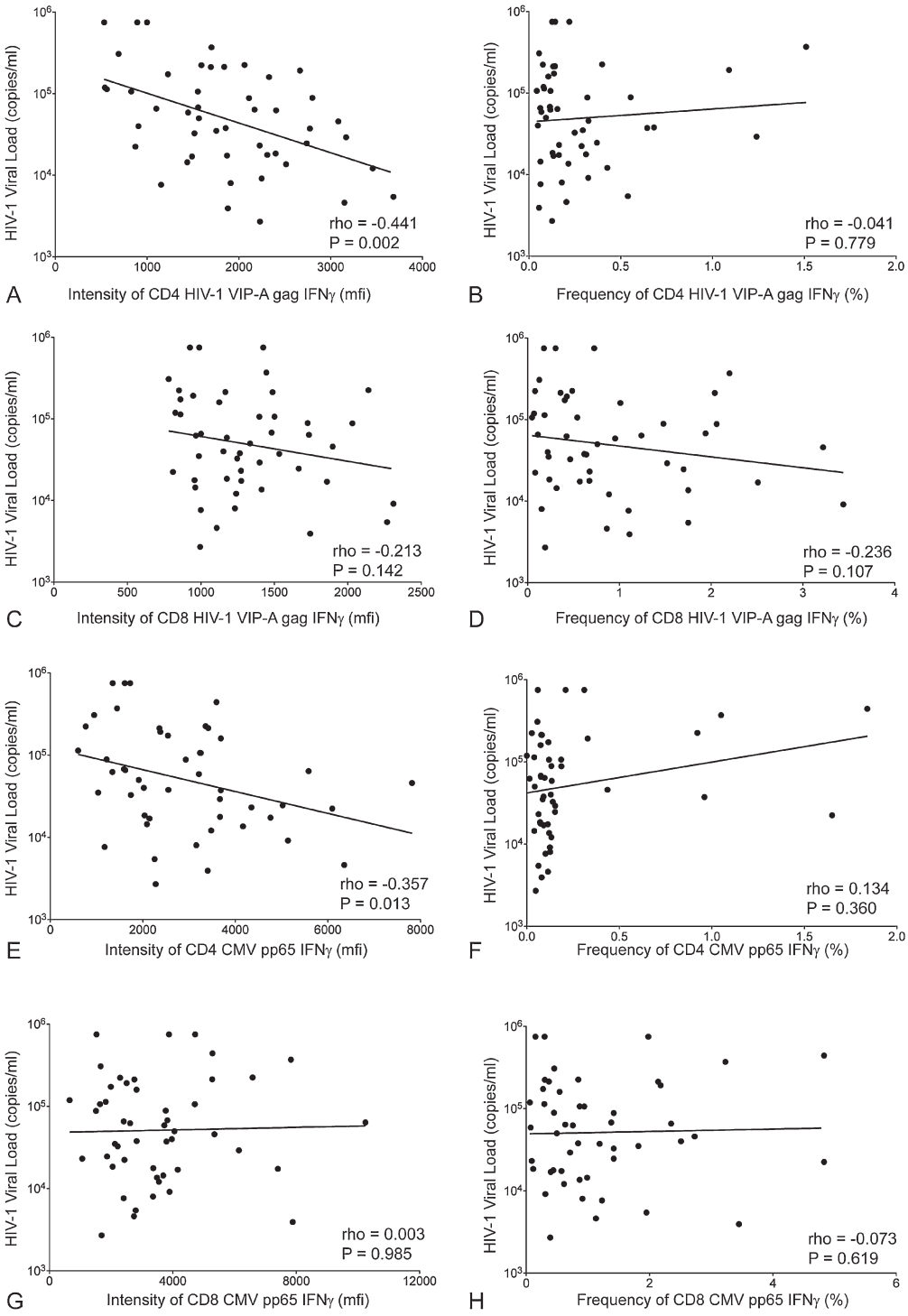

FIGURE 2. Associations between viral load and T-cell responses in HIV-1 subtype A infection. A, Spearman rank correlation between HIV-1 viral load and geometric mean fluorescence intensity (MFI) of the HIV-1 subtype A VIP Gag-specific CD4 T-cell IFN-γ production. B, Spearman rank correlation between HIV-1 viral load and percentage of CD4 T cells producing IFN-γ in response to the Gag VIP set. C, Spearman rank correlation between HIV-1 viral load and MFI of the Gag-specific CD8 T-cell IFN-γ production. D, Spearman rank correlation HIV-1 viral load and percentage of CD8 T cells producing IFN-γ in response to the HIV-1 subtype A Gag VIP set. E, Spearman rank correlation between HIV-1 viral load and MFI of the CMV-specific CD4 T-cell IFN-γ production. F, Spearman rank correlation between HIV-1 viral load and percentage of CD4 T cells producing IFN-γ in response to the CMVpp65 peptide pool. G, Spearman rank correlation between HIV-1 viral load and MFI of the CMV-specific CD8 T-cell IFN-γ production. H, Spearman rank correlation HIV-1 viral load and percentage of CD8 T cells producing IFN-γ in response to the CMVpp65 peptide pool.

54%–100%) of responding cells having this phenotype (Fig. 3A). When CD8 T-cell responses were interrogated similarly, there was considerable variation in CD45RO expression on the responding cells. CD8 $T_{Effector}$ cells represented a median of 45% (range: 0%–90%), whereas CD8 T_{EM} represented a median of 54% (range: 9%–100%) of the total Gag-specific CD8 T-cell population (Fig. 3B). We integrated both values by taking the ratio of CD8 $T_{Effector}$ to T_{EM} populations and observed a median ratio of 0.84 (range: 0–10) for HIV-1 subtype A Gag-specific responses (Fig. 3C).

To assess the relationship to HIV-1 disease progression, we examined the maturational status of Gag-specific CD4 and CD8 T-cell responses. The frequency of Gag-specific CD8 $T_{Effector}$ and CD8 T_{EM} cells showed no significant relationship to HIV-1 viral load ($\rho = -0.179$; $P = 0.224$ and $\rho = 0.157$; $P = 0.286$, respectively). When looking at Gag-specific CD8

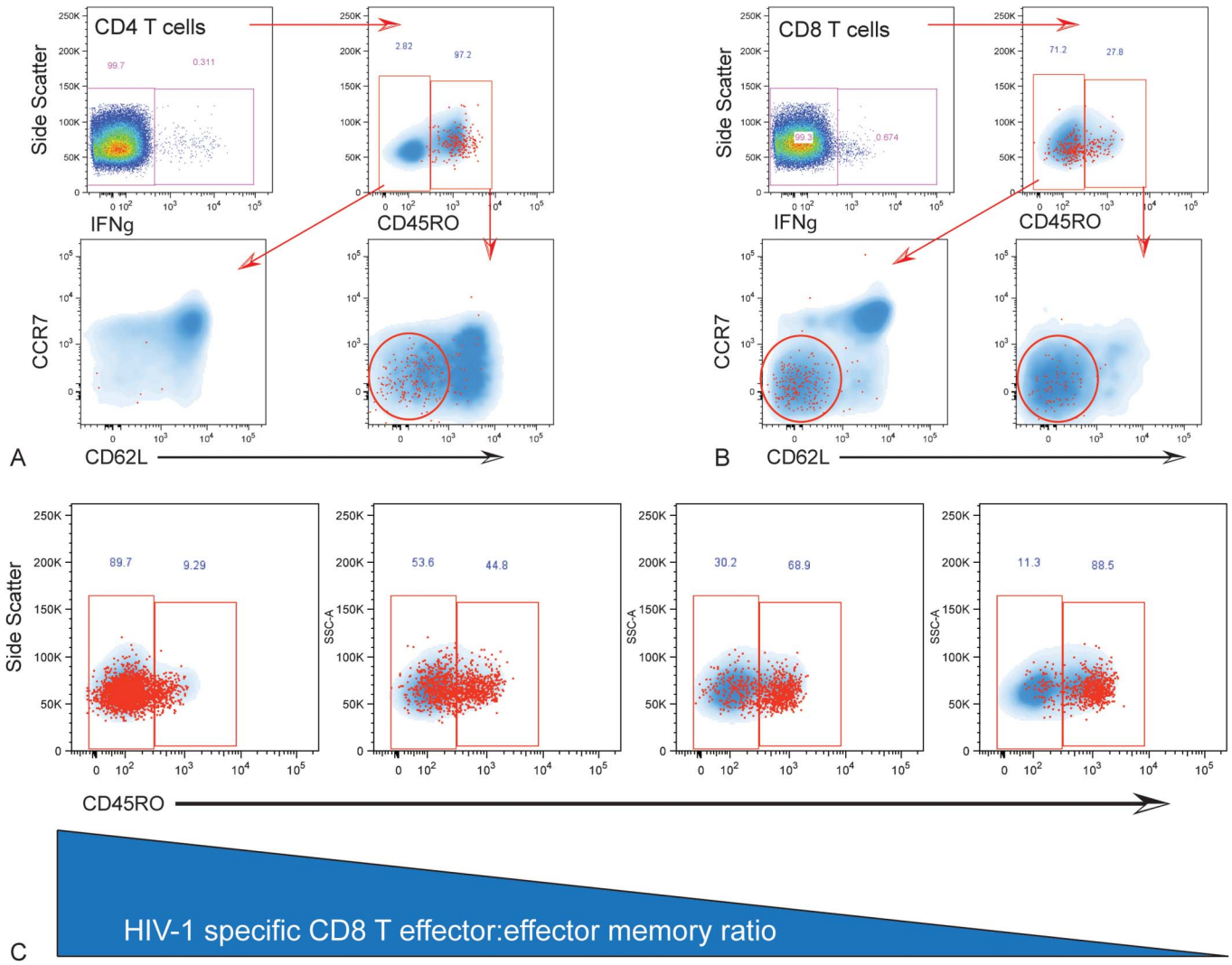

FIGURE 3. HIV-1 subtype A Gag VIP-specific T-cell responses display effector and effector memory phenotypes. PBMC from 48 HIV-1 subtype A, chronically infected, and treatment-naive, individuals were thawed and used in a modified polychromatic flow cytometry assay to characterize both phenotype (CD45RO, CCR7, and CD62L) and function (IFN-γ, IL-2, and TNF-α) in response to the HIV-1 subtype A Gag VIP set. A, Representative polychromatic histogram showing HIV-1 subtype A Gag-specific CD4 T-cell IFN-γ response and gating strategy. Positive responses were then overlaid as dot plots onto a density plot of the overall CD4 compartment with regard to CD45RO expression. Positive and negative gates were further discriminated based on CCR7 and CD62L to define memory populations with HIV-1–specific responses overlaid in red. B, Representative polychromatic histogram showing HIV-1 subtype A Gag-specific CD8 T-cell IFN-γ response and gating strategy. Positive responses were then overlaid as dot plots onto a density plot of the overall CD8 compartment with regard to CD45RO expression. Positive and negative gates were further discriminated based on CCR7 and CD62L to define memory populations with HIV-1 specific responses overlaid in red. C, A clear gradation of CD45RO expression was observed for the HIV-1–specific CD8 T-cell responses with 4 representative overlays presented. HIV-1–specific CD8 T-cell responses could then be represented as a ratio of effector (T_E) to effector memory (T_{EM}) phenotype.

$T_{Effector}$ to T_{EM} ratio, we again observed no significant relationship with the HIV-1 viral load ($\rho = -0.165$; $P = 0.261$). In addition, neither the frequency of HIV-1–specific CD8 $T_{Effector}$ and CD8 T_{EM} populations ($\rho = 0.269$; $P = 0.062$ and $\rho = -0.259$; $P = 0.073$) respectively, nor the CD8 $T_{Effector}$ to T_{EM} ratio ($\rho = 0.263$; $P = 0.068$) showed a statistically significant relationship to absolute CD4 T-cell counts. The frequency of HIV-1 subtype A Gag-specific CD4 T_{EM} showed no statistically significant relationship with HIV-1 viral load ($\rho = -0.228$; $P = 0.116$) but did show a statistically significant inverse relationship with absolute CD4 T cells counts ($\rho = -0.320$; $P = 0.025$).

Polyfunctionality in the T-Cell Response Against Subtype A VIP Sets

To examine the relative frequency of CD4 and CD8 T cells with multiple or single functions, we performed a Boolean analysis of the functions measured (CD107a, IFN-γ, IL-2, MIP-1β and TNF-α) in T cells responding to stimulation with HIV-1 subtype A Gag VIP set. CD8 T-cell responses were most frequently single MIP-1β–producing or trifunctional CD8 T cells responding with CD107a, IFN-γ, and MIP-1β (Fig. 4A). Interestingly, no significant difference was observed between the 2 distinct groups of participants with HIV-1 viral load above 100,000 copies per milliliter (n = 9) and HIV-1 viral load below 10,000 (n = 7). Within the CD4 T-cell population, Gag-specific responses were most frequently single IFN-γ–producing or CD4 T cells responding with only MIP-1β (Fig. 4B). Similarly, participants with high HIV-1 viral load above 100,000 copies per milliliter (n = 9) and those with low HIV-1 viral load below 10,000 copies per milliliter (n = 7) showed no statistically significant difference, although participants with high viral load showed a trend toward a decrease in IFN-γ–producing subsets and an increased monofunctional MIP-1β–producing population.

DISCUSSION

Clarity regarding the immune mechanisms involved in HIV-1 control may lead to more effective strategies for the prevention and treatment of HIV-1 infection. To this end, we characterized CD4 and CD8 T-cell responses to HIV-1 subtype A infection, which is associated with slower disease progression.[6–9] Using Gag-derived, Nef-derived, and gp41-derived VIP sets covering a majority of the known diversity within HIV-1 subtype A, functional and phenotypic profiles were analyzed. CD8 T cells exhibited consistent functional profiles (CD107a, IFN-γ, and MIP-1β production) against all 3 VIP Gag, gp41, and Nef sets. However, the frequency, magnitude, phenotype, and quality of the CD8 T-cell response showed no relationship to viral load or CD4 T-cell absolute count. In contrast, CD4 T-cell responses focused primarily on the Gag VIP set and were dominated by IFN-γ production. CD4 T cells responding to HIV-1 Gag displayed an effector memory phenotype, and notably, the amount of IFN-γ produced per cell was inversely proportional to viral load. Interestingly, a similar pattern was observed for CMV-specific CD4 T-cell responses. Thus, in chronic subtype A infection, the response potency at the single-cell level, but not frequency, of both Gag-specific and CMV-specific CD4 T-cell immune response may be influential in viral control or representative of a desirable immune status.

Evidence exists that support a role for CD4 T cells in viral control. Several studies have shown association of CD4 T-cell proliferation with control of HIV-1.[21,22,40] Conversely, progressive HIV-1 infection has been associated with diminished IL-2 production and a dominant IFN-γ–producing phenotype.[23] We observed a trend toward higher monofunctional MIP-1β–producing Gag-specific CD4 T cells in individuals with higher viral loads and a trend toward more IFN-γ–producing monofunctional Gag-specific CD4 T cells in individuals with lower viral loads. The presence of polyfunctional CD4 T cells is proposed to be favorable, and the intensity of IFN-γ production is predictive of the multifunctional capacity.[1,41,42,43] Our data, however, do not support an argument in favor of multifunctional CD4 T cells. It is important to note that our assay only allows for the detection of 5 functions, and other functions such as the costimulatory capacity mediated via CD40 ligand may be involved.

CD4 T cells also possess cytotoxic functions that may be directly involved in HIV-1 control. Norris et al[44] showed that HIV-1–specific CD4 T cells were able to lyse p24 peptide–pulsed autologous transformed B cells through perforin-mediated mechanisms. In the nonhuman primate model, Gag-specific and Nef-specific CD4 T cells demonstrated cytotoxic ability against simian immunodeficiency virus-infected macrophages.[45] Additionally, degranulation and CD40 ligand expression can dominate the Gag-specific CD4 T-cell response in long-term nonprogressors.[24] Interestingly, we observed relatively little or no CD4 T-cell degranulation in response to VIP Gag, Nef, and gp41 sets nor with the CMVpp65 peptide set. This difference might be explained by the geographic and host genetic disparity from the respective populations or from the HIV disease or treatment status between studies.

Our data indicate that the quality of the Gag-specific and CMV-specific CD4 T-cell IFN-γ response at the single-cell level is associated with the control of HIV-1 viral load. This may suggest a direct or indirect antiviral role of CD4 T cells in the control of HIV replication. However, it is also possible that vigorous CD4 T-cell function measured this way is reflective of the general quality of immune health, and perhaps a consequence of low viral load as opposed to an indication of active participation in the reduction of HIV-1 viremia. It is interesting to speculate that preservation of strong Gag-specific and CMV-specific CD4 T-cell responses may result from differential susceptibility of pathogen-specific CD4 T cells to HIV infection, consistent with a recent report where *Mycobacterium tuberculosis*–specific CD4 T cells were preferentially infected and depleted compared with CMV-specific CD4 T cells.[46] In addition to general immune health, host genetic determinants could be responsible for the higher levels of CD4 T-cell function observed in the present study. Studies evaluating HIV-1 controllers demonstrate higher avidity CD4 T cells and individuals with certain human leukocyte antigen class II alleles display stronger CD4 T-cell responses compared with HIV-1 noncontrollers.[47,48] In future studies, it will be important to study host factors such

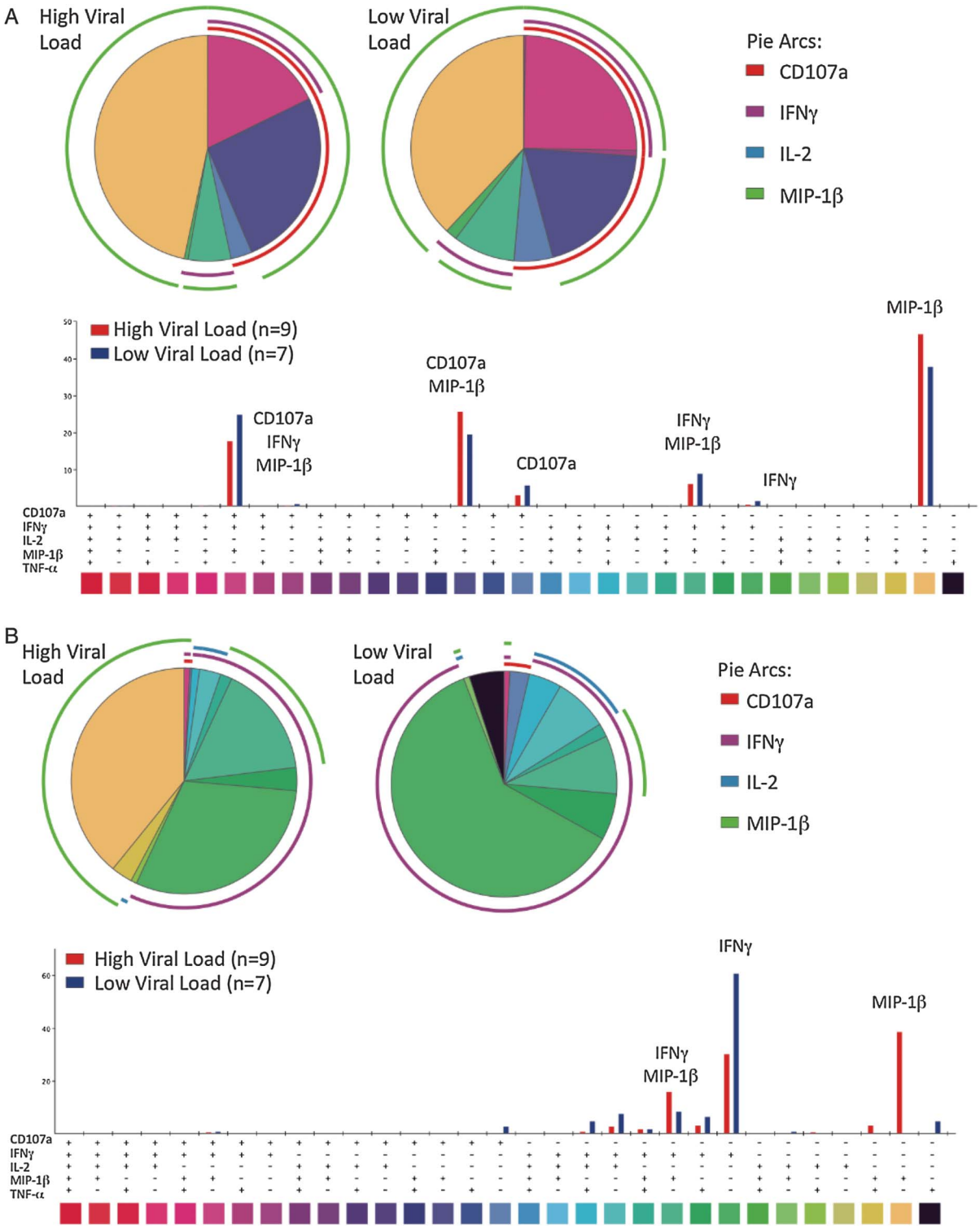

FIGURE 4. Polyfunctional analysis of CD4 and CD8 T cells responding to the HIV-1 subtype A Gag VIP set. SPICE software (version 5.1) was used to graphically present responses against HIV-1 subtype A Gag (with unstimulated responses subtracted out) for patients with high viral load (> 100,000 copies/mL; n = 9) and patients with low viral load (< 10,000 copies/mL; n = 7). Pie charts show the distribution of functional profiles corresponding to the Boolean subsets in the bar graph below for CD8 T cells (A) and CD4 T cells (B). Pie arcs showing the relative amount of each individual function: CD107a (pink), IFN-γ (purple), IL-2 (blue) and MIP-1β (green). Bar chart shows the possible 31 combinations of 5 functions on the *x*-axis and the percentage of distinct functional populations within the CD4 T cells (A) and CD8 T cells (B) on the *y*-axis.

as specific human leukocyte antigen-class II alleles in relation to the CD4 T-cell cytokine production at the single-cell level.

Although CD4 T cells are primary targets of HIV-1 and their loss is associated with disease progression, they are less well characterized in terms of the functional role they play through the course of natural infection compared with CD8 T cells. We found that the quality of the CD4 T-cell IFN-γ response per cell was associated with control of viral load in chronic HIV-1 subtype A infection. Despite observing individuals with polyfunctional CD8 T cells and disparate T_E to T_{EM} ratios, there was no significant correlation with viral burden. Use of VIP sets highlighted the effectiveness of this novel tool, as we were able to measure cellular immune responses in the majority of subjects tested regardless of viral load and CD4 count. Our data highlight a possible link between CD4 T-cell function and a better clinical outcome. Future considerations should be made to address the exact functional role of these CD4 T cells and to potentially design vaccine or treatment strategies to incorporate expansion or generation of these cells.

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