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J Immunol 2006; 176:6973-6981; ;
http://www.jimmunol.org/content/176/11/6973

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Maintenance of HIV-Specific CD4$^+$ T Cell Help Distinguishes HIV-2 from HIV-1 Infection[1]

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Unlike HIV-1-infected people, most HIV-2-infected subjects maintain a healthy CD4$^+$ T cell count and a strong HIV-specific CD4$^+$ T cell response. To define the cellular immunological correlates of good prognosis in HIV-2 infection, we conducted a cross-sectional study of HIV Gag-specific T cell function in HIV-1- and HIV-2-infected Gambians. Using cytokine flow cytometry and lymphoproliferation assays, we show that HIV-specific CD4$^+$ T cells from HIV-2-infected individuals maintained proliferative capacity, were not terminally differentiated (CD57$^-$), and more frequently produced IFN-γ or IL-2 than CD4$^+$ T cells from HIV-1-infected donors. Polyfunctional (IFN-γ^+/IL-2$^+$) HIV-specific CD4$^+$ T cells were found exclusively in HIV-2$^+$ donors. The disparity in CD4$^+$ T cell responses between asymptomatic HIV-1- and HIV-2-infected subjects was not associated with differences in the proliferative capacity of HIV-specific CD8$^+$ T cells. This study demonstrates that HIV-2-infected donors have a well-preserved and functionally heterogeneous HIV-specific memory CD4$^+$ T cell response that is associated with delayed disease progression in the majority of infected people. *The Journal of Immunology,* **2006, 176: 6973–6981.**

The HIV-1 epidemic continues to spread worldwide, and an effective vaccine to prevent infection remains elusive. A major barrier to HIV vaccine development is a limited understanding of the key components of protective immunity against HIV infection. To define these components, considerable attention has been given to the minority of HIV-1-infected individuals who control viral replication and remain asymptomatic for 10 years or more (1–3), so-called "long-term nonprogressors" (LTNPs),[3] who account for no more than 2–5% of HIV-1-infected cohorts. Fewer studies have addressed the relationship between the immune response and control of viral replication with clinical outcome in HIV-2 infection. The clinical course of HIV-2 infection is distinct from that of HIV-1. Despite a close phylogenetic relationship between the viruses, mortality rates are substantially lower in HIV-2- than in HIV-1-infected populations (4, 5). Although many HIV-2-infected individuals experience nonprogressive disease (4), a minority develop higher levels of plasma viral load and CD4$^+$ T cell decline (6), leading to an immunodeficiency syndrome indistinguishable from AIDS caused by HIV-1. Thus, most HIV-2-infected individuals behave as LTNPs although in a minority of cases the virus is capable of causing profound immunodeficiency and death.

Recent evidence suggests that many HIV-2 isolates are significantly less fit than group M HIV-1 isolates in terms of replicative and transmission capacity (7). However, some HIV-2 isolates have been shown to replicate well in PBMC (7–9) and deplete CD4$^+$ T cells in ex vivo lymphoid tissue cultures (10). The proviral burden of HIV-2 is similar to that of HIV-1 in individuals at equivalent stages of disease (11–13). However, the plasma viral load is significantly lower in most HIV-2-infected individuals (14). This presumably contributes to substantially lower rates of vertical and horizontal HIV-2 transmission (15, 16). Collectively, these data suggest that a different dynamic equilibrium of host and virus is established during HIV-2 infection, raising the possibility that control of viral replication may in part be due to a more efficient host immune response to HIV-2 (17).

Studies of the cellular immune response to HIV-2, though limited in number, have demonstrated vigorous HIV-2-specific cytotoxic T cell responses in HIV-2-infected subjects (18, 19). In addition, more recent studies have shown that HIV-1-and HIV-2-specific CD8$^+$ T cells have similar capacity to produce IFN-γ and proliferate (20, 21), suggesting that enhanced viral control in HIV-2-infected people is not associated with these aspects of the virus-specific CD8$^+$ T cell response. Although some HIV-2-infected individuals have been shown to mount a CD4$^+$ T cell response (22–25), there have been no studies to date investigating multiple functional and phenotypic parameters of HIV-2-specific CD4$^+$ T cells.

In this study, we examined T cell immune responses to HIV-2 and HIV-1 in a well-characterized group of individuals attending a clinic in The Gambia, West Africa (Table I). In a cross-sectional analysis of 39 HIV-1$^+$ and 33 HIV-2$^+$ donors stratified by CD4$^+$ T cell count, we quantified the HIV-specific CD4$^+$ T cell response by intracellular cytokine staining (ICS) for IFN-γ and IL-2 and examined surface expression of CD57 for evidence of terminal differentiation. In addition, we monitored the proliferative capacity of

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Received for publication December 29, 2005. Accepted for publication March 10, 2006.

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[3] Abbreviations used in this paper: LTNP, long-term nonprogressor; ICS, intracellular cytokine staining; ART, antiretroviral therapy; SEB, staphylococcal enterotoxin B.

0022-1767/06/$02.00

Table I. *Clinical and demographic characteristics of study cohort*[a]

Subjects	*n*	CD4 Percentage Mean (range)	Absolute CD4 Count Mean cells/ml (range)	Plasma Viral Load Mean RNA copies/ml (range)	Age Mean (range)	Sex
HIV-1						
Asymptomatic	11	34 (28–48)	760 (410–1,610)	18,973 (<100–127,933)[b]		
Intermediate	13	21 (15–26)	546 (300–960)	51,356 (661–317,944)		
AIDS	15	9 (2–13)	239 (20–550)	84,423 (16,346–151,537)		
Total	39				34 (2–66)	23/39 (59%) Female
HIV-2						
Asymptomatic	17	36 (29–52)	701 (310–1,440)	1,485 (<100–5,910)[c]		
Intermediate	10	21 (15–25)	448 (200–640)	10,754 (<100–77,430)[c]		
AIDS	6	9 (4–13)	137 (70–240)	260,312 (47,628–996,541)		
Total	33				37 (17–61)	24/33 (77%) Female
HIV negative						
Total	21	43 (33–52)	1,011 (537–2,133)		43 (33–52)	5/21 (24%) Female

[a] $CD4^+$ percentage is the percentage of total peripheral lymphocytes that are $CD3^+$ $CD4^+$. Asymptomatic category includes individuals with $CD4^+$ percentages within the normal range (≥28%). Intermediate category includes individuals with intermediate CD4 percentage (14–27%). AIDS category includes symptomatic individuals with clinical AIDS (CD4% <14). Viral load (VL) <100 = below the limit of detection. Individuals with undetectable VL were assigned a VL at the level of detection (100 RNA copies/ml) for calculation of mean.

[b] One individual with undetectable VL.

[c] Three individuals with undetectable VL.

HIV-specific $CD4^+$ and $CD8^+$ T cells. We show that nonprogression in HIV-2 infection is associated with strong HIV-2-specific $CD4^+$ T cell responses, particularly a non-terminally differentiated, polyfunctional population of cytokine-producing cells with preserved proliferative capacity.

Materials and Methods

Study population

This study was conducted at the Medical Research Council (MRC) Laboratories in Fajara, The Gambia, West Africa. A total of 72 HIV-infected individuals, 39 HIV-1^+ and 33 HIV-2^+, was recruited from the clinical HIV cohort attending the Genitourinary Medicine Clinic based at the MRC Unit. Additionally, 21 confirmed HIV-seronegative blood donors were recruited from the National Blood Bank at the Royal Victoria Teaching Hospital in Banjul, The Gambia. As antiretroviral therapy (ART) was not available in The Gambia until recently, all study subjects in this study were untreated. All individuals recruited for this study were infected with either HIV-2 or HIV-1 and were seronegative for the other virus. Individuals with dual HIV-1^+HIV-2^+ infection were excluded from the study. For analysis, study subjects were stratified into three categories based on $CD4^+$ T cell percentage rather than absolute CD4 count as this has proven to be a more reliable indicator of HIV-induced lymphopenia and mortality in the developing world (20, 26, 27). These stratifications included individuals with a CD4 percentage (CD4%) within the normal range (CD4% ≥28), symptomatic individuals who had an intermediate CD4% (CD4% = 14–27), and those individuals with clinical AIDS with a CD4% <14%. Percentages of $CD4^+$ T lymphocytes were determined by flow cytometry using BD MultiTest reagents and MultiSet software (BD Immunocytometry Systems). Patients' characteristics are detailed in Table I. Study participants were screened for HIV-1 and HIV-2 using the Murex ICE HIV-1.2.0 capture enzyme immunoassay (Murex Diagnostics). All reactive samples were confirmed as HIV-1 or HIV-2 positive using two type-specific competitive ELISAs (Murex Diagnostics). HIV-1 and HIV-2 plasma viral loads were measured by an in-house reverse-transcriptase PCR assay using specific long terminal repeat primers (28). The lower limit of detection for viral load assays was 100 RNA copies/ml. Samples with PCR signals below the level of detection were assigned a viral load value of 100 RNA copies/ml. HIV-1-infected individuals consistently had a greater mean plasma viral load than HIV-2-infected individuals at CD4 count >14%, however, HIV-2-infected individuals at highly advanced stages of disease had a higher mean plasma viral load than HIV-1-infected AIDS patients (Table I). This is likely due to the small number of HIV-2-infected individuals with advanced disease and is likely inflated by two individuals with very high viral loads, >300,000 copies/ml. Additionally, HIV-2 viral load has been shown to increase during *Plasmodium falciparum* infection in parasitemic individuals (29) and concomitant malaria infection cannot be ruled out as a cause of high viral load in these HIV-2-infected individuals. Ethical approval was obtained from the Gambian Government/MRC Ethics Committee and each study participant gave informed consent before entry into the study.

Synthetic peptides

Twenty-mer peptides overlapping by 10 aa corresponding to Gag sequences (p17 and p24) of the HIV-2 subtype A consensus and HIV-1 clade A consensus strains were synthesized as free amino acids (HIV-2 (Affiniti); HIV-1 (NIBSC); >85% purity by HPLC). Previous studies have shown that the majority of HIV-1 infections in The Gambia are CRF-02, an A/G recombinant strain of which the *gag* gene is subtype A (30, 31), and HIV-2 infections are with subtype A virus (32). Lyophilized peptides were resuspended in DMSO (Sigma-Aldrich) at 50 mg/ml. The overlapping 20 mer were grouped into an HIV-1 Gag pool (33 peptides) and an HIV-2 Gag pool (37 peptides) such that the concentration of each peptide in the pool was 400 μg/ml. Small aliquots of the Gag peptide pool mixtures were stored at −80°C and peptides were used at a final concentration of 2 μg/ml/peptide in all experiments.

Monoclonal Abs and flow cytometric analysis

FITC, CFSE, PE, PerCP, and allophycocyanin were used as the fluorophores for these experiments. Unconjugated anti-human CD28 and anti-human CD49d were used as costimulatory Abs. mAbs used for the ICS assay were anti-CD57 FITC, anti-IL-2 PE, anti-CD4 PerCP, and anti-IFN-γ allophycocyanin (BD Pharmingen). CFSE (Molecular Probes), anti-CD8 PE, anti-CD3 PerCP, and anti-CD4 allophycocyanin (BD Biosciences) were used for characterization of T cell proliferative responses. Four-parameter flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences). The list-mode data files were analyzed using FlowJo (Tree Star).

ICS for production of IFN-γ and IL-2 by HIV-specific $CD4^+$ T cells

Ten to 15 ml of peripheral blood was collected via venipuncture and PBMC were isolated using Ficoll-Hypaque (Pharmacia) density centrifugation. This assay was performed only on freshly isolated PBMC and stimulation was performed as previously described (33). Briefly, PBMC were resuspended at 10^6/ml in RPMI medium supplemented with 10% heat-inactivated FCS and 1 μg/ml anti-CD28 and anti-CD49d costimulatory Abs. Due to restrictions on the number of PBMCs recovered from the small volume of venipuncture, this study was restricted to the study of HIV Gag-specific responses as this protein has been shown to be highly immunodominant in both HIV-1- and HIV-2-infected individuals (25, 34, 35). Synthetic overlapping Gag peptide pools (final concentration 2 μg/ml/peptide) were used to stimulated HIV-specific responses. HIV-1 or HIV-2 Gag peptide pools were used according to the status of the individual. These viruses share 60% amino acid homology in the Gag region (36). For each individual, a negative control containing only anti-CD28 and anti-CD49d was used to measure Ag-independent stimulation and a positive control staphylococcal enterotoxin B (SEB; final concentration 2 μg/ml) was used

to ensure that cells were responsive. The cultures were incubated for 1 h at 37°C in a 5% CO_2 incubator, followed by an additional 5 h in the presence of Brefeldin A (10 μg/ml; Sigma-Aldrich). Stimulated cells were washed, fixed/permeabilized (FACSPerm2; BD Biosciences), and stained for surface markers and cytokines of interest. Cells were then washed and resuspended in 2% paraformaldehyde in PBS for flow cytometric analysis. Responses are reported after background correction, using the anti-CD28/49d-negative control to define nonspecific responses. A threshold of 0.1% was used to define a positive HIV Gag-specific $CD4^+$ T cell response because no negative control sample showed nonspecific responses above this level and HIV Gag peptides did not induce this level of nonspecific cytokine production in seronegative donors.

$CD4^+$ and $CD8^+$ T cell CFSE proliferation assay

Briefly, freshly isolated PBMC or freshly thawed cryopreserved samples rested overnight were resuspended in PBS at a concentration of 1–2 × 10^6/ml. Cells were stained with CFSE (final concentration 0.25 μM; Molecular Probes) for 7 min in a 37°C water bath. CFSE labeling was then quenched with 3 ml of FCS and cells were washed three times with RPMI supplemented with 10% FCS. CFSE-labeled PBMC were resuspended at a final concentration of 1–2 × 10^6/ml in RPMI supplemented with 10% human AB serum (Sigma-Aldrich) and 1 μg/ml anti-CD28 and anti-CD49d costimulatory Abs for culture. PBMCs from each individual were then stimulated with overlapping Gag peptide pools (2 μg/ml/peptide), SEB (2 μg/ml), or anti-CD28 and anti-CD49d alone (1 μg/ml each) and cultured at a concentration of 1–2 × 10^6 PBMC in a volume of 1 ml in 48-well plates (Nunc) at 37°C for 5 days. PBMC were then collected and stained for surface markers of interest and fixed with 2% paraformaldehyde in PBS. CFSE-labeled cells were kept free from excessive light to avoid photobleaching and were analyzed by flow cytometry within 24 h of staining. Percentages of total $CD4^+$ or $CD8^+$ T cells that divided in response to Gag peptide stimulation were calculated using the proliferation platform of FlowJo. Values for Ag-specific responses are reported as the percent divided after subtraction of background proliferation in the unstimulated samples.

Statistical analysis

Statistical tests were performed using two-tailed χ^2 analysis, Fisher's exact test, two-sided ANOVA, Mann-Whitney *U* test, and Spearman rank correlations using GraphPad Prism 4.0a software and GenStat. Values of *p* were corrected for multiple comparisons and differences were considered statistically significant when $p < 0.05$ after correction.

Results

More HIV-2- than HIV-1-infected subjects have detectable HIV Gag-specific $CD4^+$ T cells

ICS was used to monitor production of IFN-γ and IL-2 by Ag-specific $CD4^+$ T cells. Although HIV Gag-specific $CD4^+$ T cell responses were observed in the majority of both HIV-1- and HIV-2-infected subjects, more HIV-2-infected (85%; 28 of 33) than HIV-1-infected individuals (64%; 25 of 39) had $CD4^+$ T cells producing IFN-γ in response to their respective Gag peptide pools ($p = 0.04$; Table II). The difference was more marked for IL-2-producing $CD4^+$ T cells, which were detected in 64% of HIV-2-infected compared with 36% of HIV-1-infected individuals ($p = 0.01$). Interestingly, among individuals with a relatively preserved $CD4^+$ T cell count (>28%) 94% (16 of 17) of HIV-2-infected individuals had detectable IFN-γ-producing $CD4^+$ T cells, and 53% (9 of 17) had detectable IL-2-producing $CD4^+$ T cells. In contrast, 64% (7 of 11) of asymptomatic HIV-1-infected individuals had detectable IFN-γ-producing $CD4^+$ T cells, while only 9% (1 of 11) had IL-2-producing $CD4^+$ T cells (Table II).

HIV-2-infected donors have higher frequencies of HIV-specific $CD4^+$ T cells than HIV-1-infected donors

We quantified the frequency of $CD4^+$ T cells producing IFN-γ only, IL-2 only, or both IFN-γ and IL-2 in response to stimulation with overlapping Gag peptides using flow cytometry (representative flow cytometric plots are shown in Fig. 1*a*). In donors with relatively preserved $CD4^+$ T cell counts (≥28%), a greater proportion of total $CD4^+$ T cells from HIV-2-infected subjects produced IFN-γ in response to Gag peptides than from HIV-1-infected subjects (median 0.315 vs 0.130%; $p = 0.014$) (Fig. 1*b*). Additionally, the frequency of IL-2-producing HIV-specific $CD4^+$ T cells was significantly greater in HIV-2-infected individuals with preserved $CD4^+$ T cell counts (median 0.068%) than in CD4-matched HIV-1-infected individuals (median 0.012%; $p = 0.0077$) (Fig. 1*c*). The differences in HIV-specific $CD4^+$ T cell responses between HIV-1- and HIV-2-infected subjects were less obvious in donors with intermediate $CD4^+$ T cell counts (CD4% = 14–27) and were absent in donors with CD4% <14 (Fig. 1, *b* and *c*). $CD4^+$ T cell responses to the superantigen SEB did not differ between any group of HIV-1- and HIV-2-infected individuals (data not shown) and control experiments in HIV-seronegative blood donors demonstrated that HIV Gag peptides induced <0.1% of total $CD4^+$ T cells to produce either IFN-γ or IL-2.

Overall, the frequency of cytokine-producing HIV-specific $CD4^+$ T cells was far greater in HIV-2^+ individuals with preserved $CD4^+$ T cell counts than in HIV-1^+ individuals with comparable $CD4^+$ T cell counts; individuals infected with HIV-2 had a 2.4-fold higher median frequency of IFN-γ-producing $CD4^+$ T cells, and a 5.7-fold higher median frequency of IL-2-producing $CD4^+$ T cells, when stimulated with their respective Gag peptide pools. These trends were the same when individuals were stratified by absolute CD4 count rather than by CD4%. Significantly greater frequencies of IFN-γ- or IL-2-producing HIV-specific $CD4^+$ T cells were found among HIV-2-infected individuals with an absolute CD4 count ≥500 cells/ml compared with CD4-matched HIV-1-infected individuals (data not shown). These findings indicate that asymptomatic HIV-2^+ individuals maintain a more substantial HIV-specific memory $CD4^+$ T cell response than HIV-1^+ subjects.

HIV-specific $CD4^+$ T cells producing both IFN-γ and IL-2 are detected exclusively in HIV-2-infected individuals

HIV-specific $CD4^+$ T cells that produced both IFN-γ and IL-2 in response to HIV Gag peptides were detected at a frequency of >0.1% in 36% (12 of 33) of HIV-2^+ subjects, predominantly among individuals with a normal $CD4^+$ T cell percentage (Fig. 1, *a* and *d*). This population of $CD4^+$ T cells made a substantial

Table II. *Proportion of HIV-1- and HIV-2-infected individuals with $CD4^+$ T cells producing IFN-γ or IL-2 in response to HIV Gag peptides*[a]

	IFN-γ Responses			IL-2 Responses		
	No. positive (%)	No. negative	*p*	No. positive (%)	No. negative	*p*
All HIV-2^+ ($n = 33$)	28 (85)	5	**0.04**	21 (64)	11	**0.01**
All HIV-1^+ ($n = 39$)	25 (64)	14		14 (36)	25	
Asymptomatic HIV-2^+ ($n = 17$)	16 (94)	1	0.06	9 (53)	8	**0.04**
Asymptomatic HIV-1^+ ($n = 11$)	7 (64)	4		1 (9)	10	

[a] Ag-specific responses above 0.1% of total $CD4^+$ T cells producing cytokine were considered a positive response. Statistical significance was determined using χ^2 analysis or Fisher's exact test (for tests with less than five individuals in a category) and signifcant differences are in bold type.

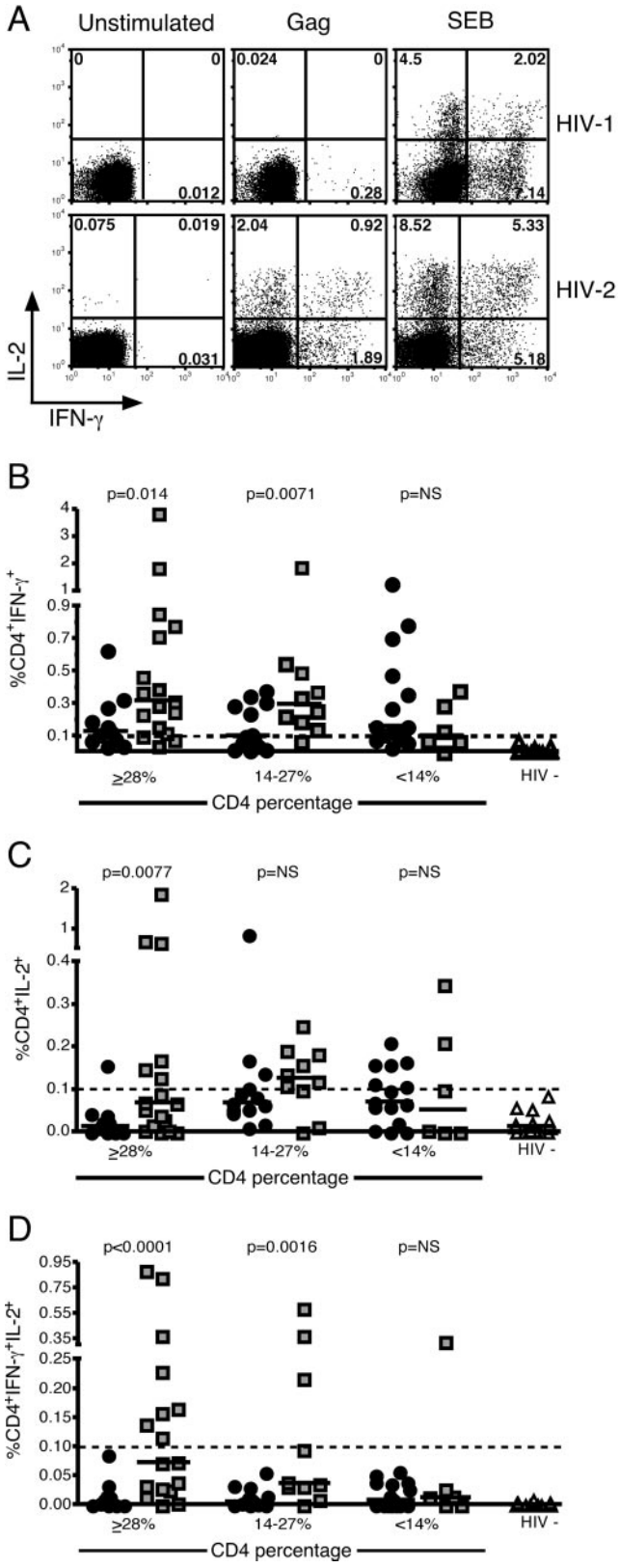

FIGURE 1. Functional flow cytometric analysis of HIV-1 and HIV-2 Gag-specific $CD4^+$ T cell response. *a*, Representative FACS plots from asymptomatic HIV-1 (*top row*) and HIV-2 (*bottom row*) subjects, both with $CD4^+$ T cell percentages ≥28%. The flow cytometric data represent gated $CD4^+$ T cells and are demonstrated as follows: background (costimulatory Abs alone, *left column*); HIV Ag-specific (Gag peptide pool, *center column*); and polyclonal positive control (SEB, *right column*). *y*-axis represents staining for IL-2 and *x*-axis is staining for IFN-γ. The numbers in each quadrant indicate the percentage of $CD4^+$ T cells that produced either IFN-γ alone (*bottom right quadrant*), IL-2 alone (*upper left quadrant*), or both IFN-γ and IL-2 (*upper right quadrant*) in response to stimulus. Scatter plots show the proportion of $CD4^+$ T cells producing IFN-γ (*b*) IL-2 (*c*) or both IFN-γ and IL-2 (*d*) in HIV-2 (□), HIV-1 (■), or HIV-negative (△) donors in response to pooled Gag peptides. Median value in each category is delineated with a horizontal bar and represents total Ag-specific responses above background. A dotted line at 0.1% response indicates the level above which responses are considered to be positive. Data were log-transformed for analysis and statistical significance was determined using ANOVA and adjusted for multiplicity using the Holm step-down method.

contribution to the total HIV-2 Gag-specific $CD4^+$ T cell response, accounting for ~17% of the response irrespective of $CD4^+$ T cell count (Fig. 2*a*). In contrast, this dual cytokine-producing population of HIV-specific $CD4^+$ T cells was absent (<0.1%) from all 39 HIV-1-infected individuals we studied (Figs. 1, *a* and *d*, and 2*b*). In our cohort of HIV-1-infected donors, HIV-1-specific $CD4^+$ T cells responded to Gag peptides by producing either IFN-γ or IL-2, but not both.

Those HIV-2-infected individuals with strong $CD4^+$ T cell responses to HIV-2 Gag peptides had high frequencies of $CD4^+$ T cells in all three functional populations: IFN-γ only, IL-2 only, and polyfunctional IFN-γ^+IL-2^+. There was a strong correlation between the frequency of $CD4^+$ T cells producing only IFN-γ and those $CD4^+$ T cells producing IL-2 alone ($R = 0.43$, $p = 0.01$; data not shown). There was also a very strong correlation between the frequency of polyfunctional IFN-γ^+IL-2^+CD4^+ T cells and the frequency of IFN-γ only ($R = 0.69$, $p < 0.0001$) or IL-2 only producing $CD4^+$ T cells ($R = 0.47$, $p = 0.005$; data not shown). This suggests that the $CD4^+$ T cell response to HIV-2 is indeed functionally heterogeneous and individuals with strong responses tend to have a total $CD4^+$ T cell response to the virus that is a compilation of at least three functionally distinct cytokine-producing populations.

The increase in HIV-specific $CD4^+$ T cells in HIV-2 infection is due to the presence of $CD4^+$ T cells lacking CD57 expression

An important function of $CD4^+$ T cells is their ability to proliferate in response to cognate Ag stimulation. Surface expression of CD57 is associated with the inability to proliferate and indicates terminal differentiation (37). To determine whether the higher frequency of HIV-specific $CD4^+$ T cells in HIV-2-infected individuals was among the terminally differentiated ($CD57^+$) or non-terminally differentiated ($CD57^-$) fraction, we measured CD57 expression on the HIV-1- and HIV-2-specific cytokine-producing $CD4^+$ T cells. We found that the increase in HIV-specific cytokine-producing $CD4^+$ T cells in HIV-2^+ as compared with HIV-1^+ subjects with preserved $CD4^+$ T cell counts was predominantly within the $CD57^-$ fraction ($p = 0.01$, Fig. 3*b*). There was no difference in the frequency of $CD57^+$ HIV-specific, cytokine-producing $CD4^+$ T cells among HIV-1- or HIV-2 infected individuals at any stage of disease (Fig. 3*a*). These data indicate that the increase in HIV-2-specific $CD4^+$ T cells is predominantly made up of $CD57^-$ cells, which likely retain proliferative capacity.

Interestingly, HIV-2-specific $CD4^+$ T cells that expressed CD57 were skewed toward IFN-γ-producing populations, with a mean 77% of the Gag-responsive $CD4^+$ T cells producing IFN-γ alone (Fig. 3*c*). HIV-2-specific $CD4^+$ T cells lacking CD57 expression were more polyfunctional in nature, with a greater percentage of $CD4^+$ T cells producing IL-2 in response to Gag peptides (Fig. 3*d*).

Superior proliferative capacity of HIV-2-specific $CD4^+$ T cells

Ag-specific $CD4^+$ T cell proliferation has been regarded as a major indicator of functional $CD4^+$ T cell help. We used the

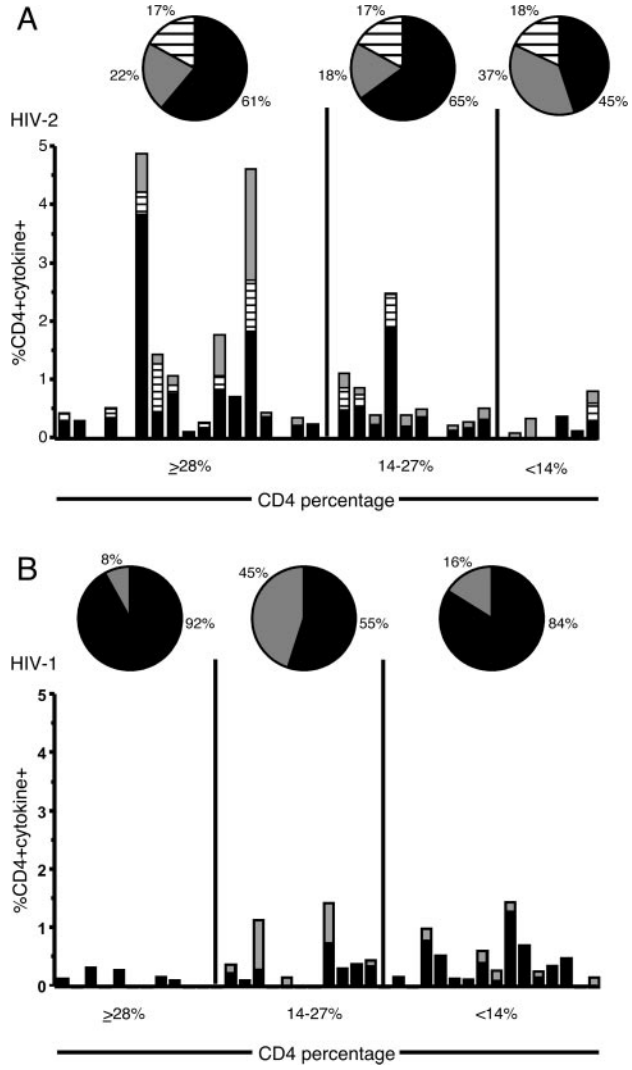

FIGURE 2. Functional heterogeneity of HIV-2-specific CD4+ T cell cytokine profile. Stacked graphs show percentage of CD4+ T cells that produced IFN-γ only (black), IL-2 only (gray), or both IFN-γ and IL-2 (striped) in response to pooled Gag peptides in the 6-h assay in HIV-2 (*a*) and HIV-1 (*b*) infected individuals. A column composed of these three segments represents each individual's response. Ag-specific responses above 0.1% of total CD4+ T cells producing each cytokine were considered a positive response. Total IFN-γ production is the combined black and striped segments. Total IL-2 production is the combination of the gray and striped segments. Vertical lines stratify patients based on CD4+ T cell percentage and pie charts depict the mean proportion of the total HIV-specific response contributed by each cytokine population in each stratified category.

CFSE-based lymphoproliferation assay to monitor $CD4^+$ and $CD8^+$ T cell proliferative capacity in response to HIV Gag peptides in HIV-1^+ and HIV-2^+ study participants. Fresh PBMC from 14 HIV-1- and 18 HIV-2-infected individuals were isolated from a venipuncture 5–10 mo after the original time point. For those individuals who were lost to follow-up, we used frozen PBMC samples where available (14 HIV-1 and 8 HIV-2 samples collected between 16 mo before and 8 mo after the original time point). We could not complete the study for 11 of 39 HIV-1-and 7 of 33 HIV-2-infected individuals from our original cohort for whom there were no frozen historical samples.

Fresh or frozen PBMC were stimulated with Gag peptides, SEB, or costimulatory Abs alone for 5 days and proliferative responses were measured in the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets (representative flow plots are shown in Fig. 4*a*). Stimulation of PBMC from HIV-2-infected individuals with normal $CD4^+$ T cell percentages (≥28%) with HIV-2 Gag peptides induced the proliferation of a median 0.6% of total $CD4^+$ T cells over 5 days (range 0.0–3.1%; Fig. 4*b*). In striking contrast, we observed a profoundly attenuated proliferative response in $CD4^+$ T cells from HIV-1-infected individuals with comparable $CD4^+$ T cell percentages. In these subjects, a median 0.2% of total peripheral blood $CD4^+$ T cells expanded in response to HIV-1 Gag peptides over 5 days (range 0.02–0.40%; $p = 0.03$). These differences were also apparent when subjects were stratified by absolute CD4 count (data not shown). Thus, the proliferative capacity of HIV-2-specific $CD4^+$ T cells among individuals with normal $CD4^+$ T cell counts was 3-fold greater than that of CD4-matched HIV-1-infected individuals. This difference was not observed in individuals with $CD4^+$ T cell percentage below the normal range (<28%) where proliferative responses in both HIV-1- and HIV-2-infected individuals were diminished.

No difference in the capacity of HIV-2- and HIV-1-specific $CD8^+$ T cells to proliferate

In contrast to the differences we observed in the proliferative capacity of HIV-specific $CD4^+$ T cells between HIV-1^+ and HIV-2^+ individuals, we found no differences in the ability of HIV Gag-specific $CD8^+$ T cells from HIV-1- and HIV-2-infected individuals to proliferate in response to type-specific HIV Gag peptides (Fig. 4*c*). The median frequencies of responding $CD8^+$ T cells were similar among individuals with a normal $CD4^+$ percentage, with responses of 0.60% in HIV-2^+ subjects compared with 0.89% in HIV-1^+ donors ($p = 0.34$). The frequency of proliferating $CD8^+$ T cells decreased as CD4 counts declined in both HIV-1- and HIV-2-infected individuals, but the median frequencies still did not differ significantly (0.17 vs 0.09%; $p = 0.99$).

The frequency of polyfunctional IFN-γ^+IL-2^+ $CD4^+$ T cells correlates with the proliferative capacity of HIV-2-specific $CD4^+$ and $CD8^+$ T cells

Previous studies have shown that HIV-1-infected individuals who fail to control viral load even in the presence of ART have HIV-specific $CD4^+$ T cells that predominantly produce IFN-γ, and have a greatly diminished proliferative capacity (38, 39). In contrast, we find that HIV-2^+ individuals have an expanded frequency of polyfunctional IFN-γ^+IL-2^+CD4^+ T cells which correlated with the proliferative capacity of both HIV-2-specific $CD4^+$ ($R = 0.44$, $p = 0.02$) and $CD8^+$ ($R = 0.44$, $p = 0.02$) T cells (data not shown). We also found that although HIV-2-infected individuals with a high frequency of HIV-specific IFN-γ^+IL-2^+CD4^+ T cells tended to have low or undetectable plasma viral load, there was no correlation between these parameters (data not shown).

Discussion

Although HIV-2 can, in a minority of infected individuals, cause AIDS indistinguishable from that caused by HIV-1, many HIV-2-infected people behave as LTNPs. This provides a unique natural human model in which to investigate correlates of immune protection against HIV disease progression. One of the characteristic immune defects in HIV-1 infection is the early loss of HIV-specific $CD4^+$ T cells with proliferative capacity and the ability to secrete IL-2 (3, 40), which leaves an impaired $CD4^+$ Th cell response that is skewed toward a population that produces IFN-γ only (33, 39). Indeed, aside from a unique population of HIV-1-infected individuals who meet the definition of LTNPs and some patients treated with highly active ART, HIV-specific $CD4^+$ T cell

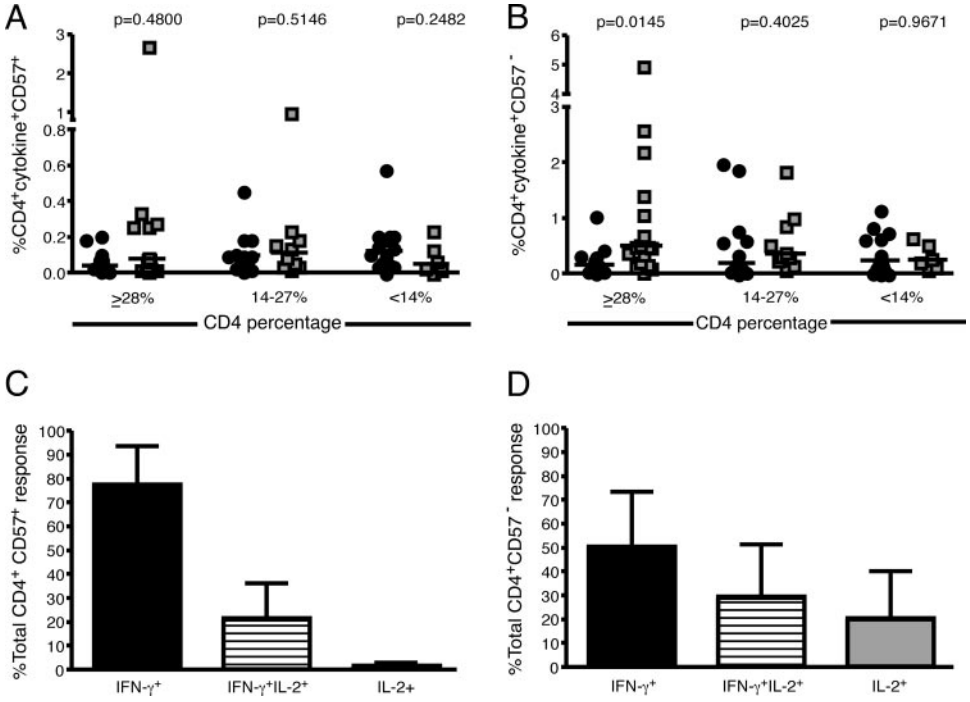

FIGURE 3. CD57 expression on HIV-specific $CD4^+$ T cells. Scatter plots show the proportion of total $CD4^+$ T cells that produced cytokine (IFN-γ^+, IL-2^+, or IFN-γ^+IL-2^+) in response to pooled Gag peptides and express (*a*) or lack (*b*) cell surface expression of CD57 in HIV-2 (□) and HIV-1 (●) donors. Median value in each category is delineated with a horizontal line. Values of *p* were generated using two-tailed Mann-Whitney *U* test. Bar graphs depict the proportion of the total HIV-2-specific $CD57^+CD4^+$ (*c*) or $CD57^-CD4^+$ (*d*) response contributed by IFN-γ^+ (■), IL-2^+ (□) or IFN-γ^+IL-2^+ (▤) populations in four HIV-2^+ individuals with the percentage of $CD4^+$cytokine$^+CD57^+$ >0.28%. Mean and SDs are shown.

responses are conspicuously absent or impaired in HIV-1 infection, irrespective of viral load and $CD4^+$ T cell count (1, 38, 39). In this study, we demonstrate that HIV-2-infected subjects, especially those with preserved $CD4^+$ T cell counts, are distinct from HIV-1-infected individuals in that they maintain a proliferation-competent, nonterminally differentiated, multiple cytokine-expressing HIV-specific $CD4^+$ T cell response.

These findings extend previous studies that demonstrated preserved virus-specific proliferative responses in a small number of HIV-2-infected individuals (23, 24). Although a prior study has shown that the magnitude of the HIV Gag-specific IFN-γ response by ex vivo ELISPOT was not significantly different in HIV-1- and HIV-2-infected donors (25), we found using ICS that the magnitude of $CD4^+$ T cells responding to HIV Gag peptides was much greater among HIV-2-infected individuals.

HIV-specific $CD4^+$ Th cell function is reported to be impaired from the earliest stages of HIV-1 infection (40), in the absence of prompt treatment with ART (2), which may be explained by the preferential infection of HIV-1-specific $CD4^+$ T cells (41). The devastating impact of primary SIV infection on the $CD4^+$ T cell population in gut-associated lymphoid tissue, which accounts for the bulk of the body's $CD4^+$ T cells, has recently been documented in animal models (42, 43), and a similar phenomenon is likely to occur in human HIV-1 infection, in which gut-associated lymphoid tissue $CD4^+$ T cells are profoundly depleted even in primary HIV-1 infection (44, 45). The preservation of virus-specific T cell help in many subjects with HIV-2 infection suggests that HIV-2 may not preferentially infect or deplete HIV-2-specific cells in these subjects. Further studies to investigate whether HIV-2-specific $CD4^+$ T cells resist viral infection and, if so, by what mechanism are clearly warranted.

The critical features of $CD4^+$ T cell help that contribute to protective immunity probably differ among viral infections, but recent studies in other chronic viral infections have shown that Ag load and persistence dictate the functional profile of $CD4^+$ T cell responses, and that protective and effective responses, typified by those seen in CMV and EBV infections, are characterized by the presence of $CD4^+$ T cells that produce both IFN-γ and IL-2 (46). In keeping with these observations, we find that maintenance of a normal $CD4^+$ T cell count in HIV-2 infection is associated with a population of polyfunctional IFN-γ^+IL-2^+ HIV-specific $CD4^+$ T cells that was absent in all HIV-1-infected individuals in our cohort. This polyfunctional $CD4^+$ T cell population contributes substantially (17–18%) to the total HIV-2-specific $CD4^+$ T cell response and the magnitude of this population correlates with the ability of both HIV-2-specific $CD4^+$ and $CD8^+$ T cells to proliferate in vitro. In a cross-sectional study of this nature, it is not possible to determine whether preserved $CD4^+$ T cell counts in the presence of virus-specific IFN-γ^+IL-2^+CD4^+ T cells is a cause or effect of this population. However, because this polyfunctional subset is found only among HIV-2-infected individuals, and has been shown to be a hallmark of effective immunity in other viral infections, the presence of this subset may be a contributing factor in the attenuated clinical phenotype of HIV-2 infection. Though polyfunctional IFN-γ^+IL-2^+ HIV-1-specific $CD4^+$ T cells have been documented in rare HIV-1 LTNP (47), we did not detect them in our HIV-1-infected population which was not enriched for LTNP and may have predominantly comprised donors with typical disease progression at an early stage of infection.

Terminally differentiated $CD57^+$ T cells are often IFN-γ-producing effector cells (48) that lack proliferative capacity and die upon secondary stimulation (37). In mice, these IFN-γ-producing effector cells are not precursors of memory $CD4^+$ T cells, which instead make IL-2 and proliferate on secondary stimulation (49). Consistent with this paradigm, we found that HIV-2-specific $CD57^+CD4^+$ T cells generally lacked IL-2 production and instead predominantly made IFN-γ. However, the HIV-2-specific $CD57^-CD4^+$ T cells were more polyfunctional, with 29% producing IFN-γ and IL-2, and 20% producing only IL-2. We observed similar trends in the two HIV-1-infected individuals who had a substantial $CD57^+$ HIV-specific $CD4^+$ T cell response (data not shown). These data demonstrate that the difference in frequency of HIV-specific $CD4^+$ T cells between asymptomatic HIV-1- and HIV-2-infected donors is predominantly driven by the non-terminally differentiated subset, which more frequently express IL-2.

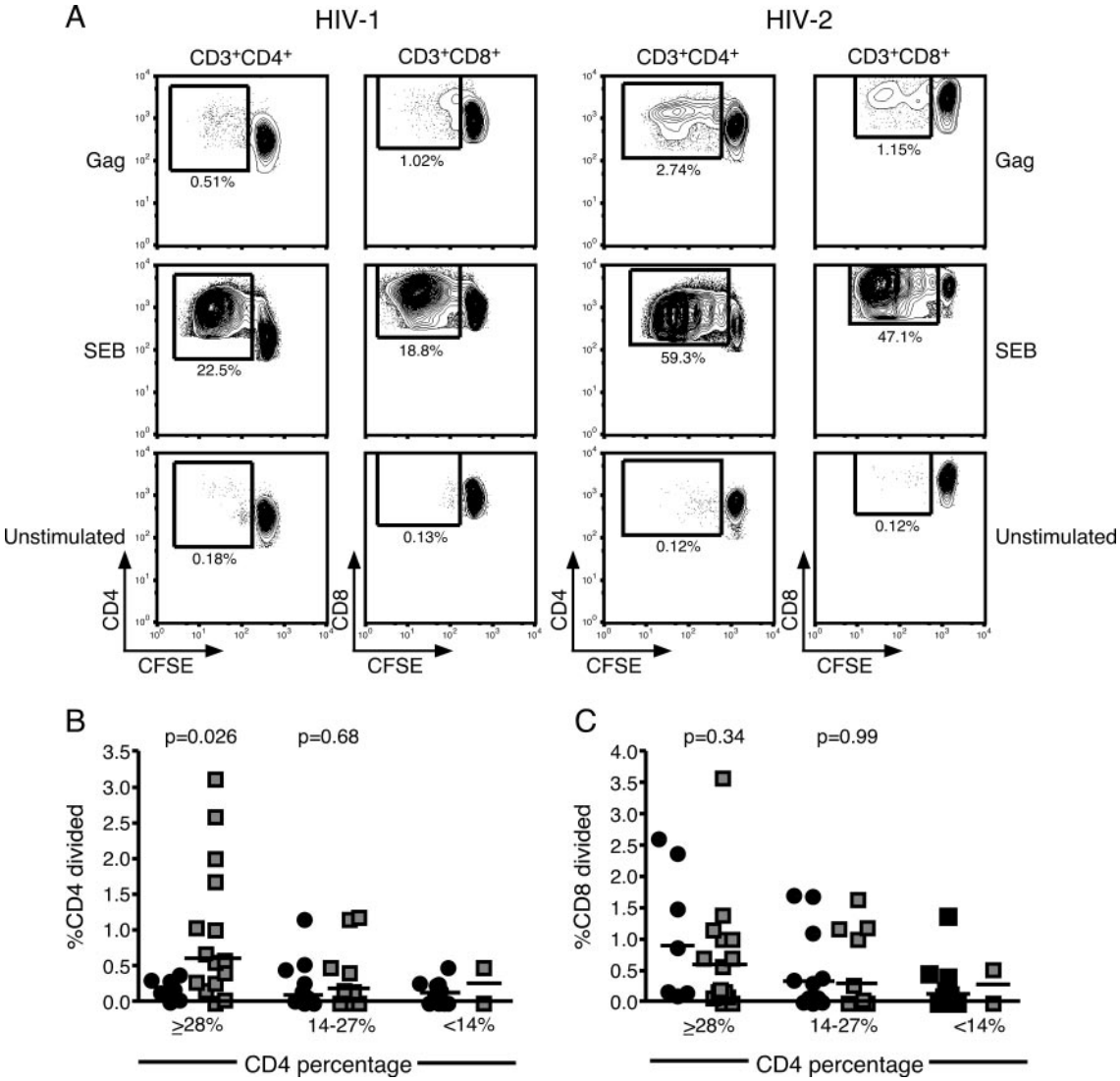

FIGURE 4. Proliferative capacity of HIV-1 and HIV-2-specific $CD4^+$ and $CD8^+$ T cells. *a*, Representative flow cytometric plots from asymptomatic HIV-1 (*left*) and HIV-2 (*right*) subjects. The flow cytometric data represent gated $CD3^+$ live lymphocytes and demonstrate HIV-specific (Gag peptide pool, *top row*), SEB (*middle row*), and background (costimulatory Abs alone, *bottom row*) stimulation conditions. $CD4^+$ T cell responses are shown in first column, $CD8^+$ T cell responses in second column, and the *x*-axis is CFSE fluorescence. The numbers indicate the percentage of each population that has divided over 5 days. Scatter plots show proportion of $CD4^+$ T cells (*b*) or $CD8^+$ T cells (*c*) dividing in response to Gag peptides in HIV-2^+ (□) or HIV-1^+ (●) donors. Median value in each category is delineated with a horizontal bar and represents total Ag-specific responses above background. Data were square-root transformed for analysis and statistical significance was determined using ANOVA.

These cells are functionally more consistent with memory than effector Th cells and should retain proliferative capacity.

Intriguingly, despite the increased functionality of HIV-2-specific $CD4^+$ T cells, we found that the maintenance of $CD4^+$ T cell help in HIV-2-infected individuals is not reflected in increased proliferative capacity of HIV-2-specific $CD8^+$ T cells. This is in keeping with other studies that have demonstrated similar antiviral activity, phenotype, and function of virus-specific $CD8^+$ T cells in HIV-1- and HIV-2-infected donors at similar stages of disease (20, 21). One potential explanation for these findings is that HIV-2-specific $CD4^+$ T cells themselves may have direct effector functions that are protective and antiviral and may be contributing to control of HIV-2 viremia via this mechanism. Alternatively, other aspects of $CD8^+$ T cell functionality that require $CD4^+$ T cell help may be enhanced in HIV-2-infected individuals. Indeed, many recent studies, mostly in murine models, have investigated the requirement of $CD8^+$ T cells for $CD4^+$ T cell help, and have suggested that $CD4^+$ T cells are required at the time of primary infection to ensure the expansion and maintenance of memory $CD8^+$ T cells in a secondary response (50). In the absence of $CD4^+$ T cell help, the secondary $CD8^+$ T cell response is both smaller (51) and functionally impaired (51, 52): that impairment is manifested in both reduced proliferative capacity and effector potential (lytic activity and IFN-γ secretion). Fewer studies have examined the role of $CD4^+$ T cell help in supporting the $CD8^+$ T cell response in persistent infection, but in the lymphocytic choriomeningitis virus model, the maintenance and function of memory $CD8^+$ T cells are impaired in the absence of $CD4^+$ T cell help (53). Consistent with these observations, impairments in HIV-1-specific $CD8^+$ T cell phenotype and function have been noted and ascribed to the lack of appropriate $CD4^+$ T cell help (54–56). Although we only measured the proliferative capacity of HIV-2-specific $CD8^+$ T cells, we found this activity to be intact in HIV-2-infected subjects who have preserved HIV-2-specific $CD4^+$ T cell function.

Other studies have failed to detect differences in the magnitude or cytokine secretion profile of HIV-specific $CD8^+$ T cells between HIV-1- and HIV-2-infected subjects (20, 21, 25). Similarly,

we did not detect significant differences in the proliferative capacity of HIV-specific $CD8^+$ T cells between HIV-1- and HIV-2-infected subjects, despite the marked discrepancy in the levels of $CD4^+$ T cell help and viral load between the two groups. Assuming that $CD8^+$ T cells are the major antiviral effector arm of the immune system, it becomes difficult to reconcile our results. However, it is certainly plausible that other aspects of $CD8^+$ T cell function than those we have measured are more effective in HIV-2 infection than in HIV-1 infection and are responsible for the differences in control of these two human pathogens. Additionally, preserved HIV-2-specific $CD4^+$ T cell help could support other host immune responses such as enhanced neutralizing Ab activity (57, 58) or improved activation of APCs.

Although our cohorts of HIV-1- and HIV-2-infected individuals were matched in terms of $CD4^+$ T cell counts (both percentages and absolute counts are similar at equivalent stages of disease), plasma viral load was >10-fold lower among asymptomatic HIV-2-infected individuals. Either as a cause or consequence of this lower viral burden, HIV-specific $CD4^+$ T cells retain a robust proliferative capacity and ability to secrete multiple cytokines. Our findings of preserved polyfunctional $CD4^+$ T cell help in HIV-2 infection should provide a foundation upon which to investigate those aspects of human $CD8^+$ T cell activity that are dependent on $CD4^+$ T cell help. Our data also provide support for vaccine strategies that are designed to elicit a strong and polyfunctional $CD4^+$ T cell response in addition to a $CD8^+$ T cell response to HIV.

Acknowledgments

We thank the medical, nursing, field, and laboratory staff of the Genitourinary Medicine Clinic in The Gambia for all their help in obtaining blood samples for this study. We thank Akum Aveika, Ramu Sarge-Njie, and Bakary Sanneh for patient recruitment and laboratory assistance. We also acknowledge the generosity of the patients from the clinic who donated these blood samples. Finally, we thank Joseph Casazza, Melissa Precopio, Jay Vasudevan, and David Price for critical review of the manuscript.

Disclosures

The authors have no financial conflict of interest.

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