



# Frequencies of Circulating Th1-Biased T Follicular Helper Cells in Acute HIV-1 Infection Correlate with the Development of HIV-Specific Antibody Responses and Lower Set Point Viral Load

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**ABSTRACT** Despite decades of focused research, the field has yet to develop a prophylactic vaccine for HIV-1 infection. In the RV144 vaccine trial, nonneutralizing antibody responses were identified as a correlate for prevention of HIV acquisition. However, factors that predict the development of such antibodies are not fully elucidated. We sought to define the contribution of circulating T follicular helper (cTfh) subsets to the development of nonneutralizing antibodies in HIV-1 clade C infection. Study participants were recruited from an acute HIV-1 clade C infection cohort. Plasma anti-gp41, -gp120, -p24, and -p17 antibodies were screened using a customized multivariate Luminex assay. Phenotypic and functional characterizations of cTfh cells were performed using HLA class II tetramers and intracellular cytokine staining. In this study, we found that acute HIV-1 clade C infection skewed the differentiation of functional cTfh subsets toward increased Tfh1 ( $P = 0.02$ ) and Tfh2 ( $P < 0.0001$ ) subsets, with a concomitant decrease in overall Tfh1-17 (which shares both Tfh1 and Tfh17 properties) ( $P = 0.01$ ) and Tfh17 ( $P < 0.0001$ ) subsets, compared to the subsets found in HIV-negative subjects. Interestingly, the frequencies of Tfh1 cells during acute infection (5.0 to 8.0 weeks postinfection) correlated negatively with the set point viral load ( $P = 0.03$ , Spearman rho [ $r$ ] =  $-60$ ) and were predictive of p24-specific plasma IgG titers at 1 year of infection ( $P = 0.003$ ,  $r = 0.85$ ). Taken together, our results suggest that the circulating Tfh1 subset plays an important role in the development of anti-HIV antibody responses and contributes to HIV suppression during acute HIV-1 infection. These results have implications for vaccine studies aimed at inducing long-lasting anti-HIV antibody responses.

**IMPORTANCE** The HIV epidemic in southern Africa accounts for almost half of the global HIV burden, with HIV-1 clade C being the predominant strain. It is therefore important to define immune correlates of clade C HIV control that might have implications for vaccine design in this region. T follicular helper (Tfh) cells are critical for the development of HIV-specific antibody responses and could play a role in viral control. Here we showed that the early induction of circulating Tfh1 cells during acute infection correlated positively with the magnitude of p24-specific IgG and was associated with a lower set point viral load. This study highlights a key Tfh cell subset that could limit HIV replication by enhancing antibody generation. This study un-

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underscores the importance of circulating Tfh cells in promoting nonneutralizing antibodies during HIV-1 infection.

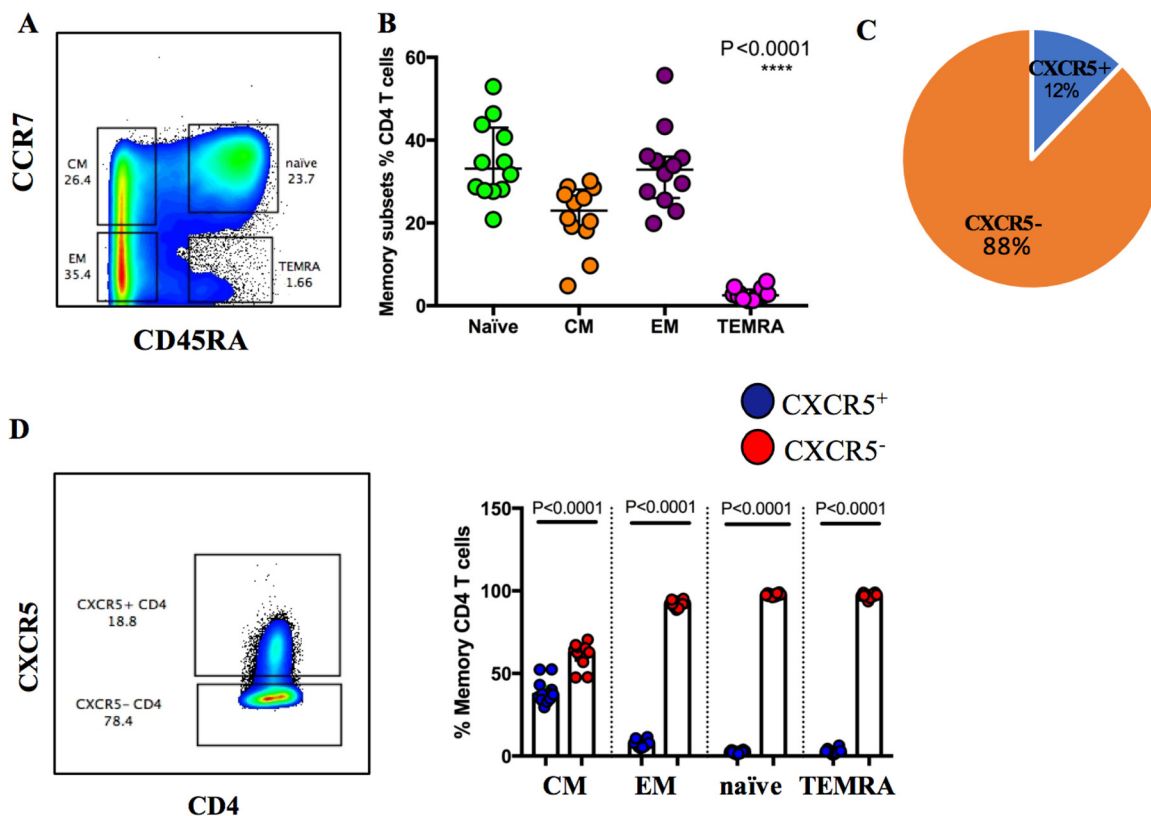
**KEYWORDS** HIV, T follicular helper cells, nonneutralizing antibodies, Gag p24 IgG, Gag p24 IgG antibodies, circulating Tfh cells

A safe and effective prophylactic vaccine remains the most efficient way of ending the human immunodeficiency virus (HIV)/AIDS epidemic, which affects over 36 million people worldwide (1). Although studies in nonhuman primate and animal models have demonstrated the efficacy of anti-HIV broadly neutralizing antibodies (bNAbs) in preventing HIV infection, human vaccine trials to date have been largely unsuccessful in inducing such responses (2–4). Thus, an improved understanding of the mechanisms that underlie the development of functional and durable anti-HIV antibody responses in the context of a natural infection will be essential for optimal vaccine design efforts (5). Moreover, with the quality of immune responses in early acute HIV infection predicting disease outcome (6, 7), early acute HIV infection is a useful model to identify early correlates of HIV-1 control.

T follicular helper (Tfh) cells, a lineage of CD4<sup>+</sup> T cells that express the chemokine receptor CXCR5, are specialized for B cell help and the development of antibody responses (8, 9). Tfh-B cell interactions in the B cell follicles promote germinal center (GC) formation, B cell differentiation, B cell survival, antibody affinity maturation, and class switch recombination (8, 10). The circulating memory counterparts of bona fide germinal center Tfh cells have been recently described (11, 12). These cells display either an activated or a quiescent phenotype based on the expression of PD-1 and ICOS or CCR7 receptors and can be further divided into subsets based on the expression of CXCR3 and CCR6 receptors (12, 13). The subsets Tfh1, Tfh2, Tfh17, and Tfh1-17 were named due to their similarities to other T helper cell lineages. Tfh1 cells express CXCR3-like Th1 cells; Tfh2 cells produce interleukin-4 (IL-4), like Th2 cells; Tfh17 cells express CCR6, similarly to Th17 cells; and Tfh1-17 cells have functional properties that are similar to those of both Th1 and Th17 cells (11–13).

From the RV144 vaccine trial, which had a modest efficacy in preventing HIV acquisition, we learned that nonneutralizing antibodies (nnAbs) could protect against HIV acquisition (14). Consistent with this observation, a recent study exploring the efficacy of nnAbs for blocking virus entry showed that anti-Env nnAbs could modulate the transmission of simian-human immunodeficiency virus (SHIV) in macaques and reduce the number of transmitted/founder viruses establishing infection in the animals (15). Moreover, a humanized mouse model of HIV infection reported the nearly complete clearance of adoptively transferred infected cells within 5 h of nnAb treatment (16), further demonstrating the potential for nnAbs in preventing HIV infection. Specific Tfh subsets have been shown to help the induction of various antibody functions. For instance, a recent study correlated the frequencies of CXCR3<sup>−</sup> circulating T follicular helper (cTfh) cells, which include both Tfh2 and Tfh17 subsets, with the development of bNAbs against HIV infection (17), suggesting a potential role of these subsets as correlates for the induction of bNAbs in infection and possibly by vaccines. It is thus important to define the specific Tfh subsets that contribute to nnAb development in the context of natural HIV infection.

Here we investigated if the induction of cTfh responses during acute HIV infection contributes to initial HIV control and promotes the development of anti-HIV nnAbs. We examined the role of HIV-specific cTfh subsets during acute HIV infection using HLA class II tetramers and multiparametric flow cytometry. HIV-specific antibody responses were further measured using a customized multivariate Luminex assay. Our results showed that acute HIV infection induces the significant expansion of HIV-specific memory Tfh1 cells ( $P = 0.02$ ), which correlated with lower set point viral loads (SPVL). Moreover, the frequencies of Tfh1 cells during early infection were predictive of p24-specific IgG titers. These data suggest that circulating Tfh1 cells play a role in



**FIG 1** Memory distribution of CXCR5+ cells within the circulating CD4+ T cell compartment in healthy donors. (A) Representative flow cytometry plot showing the gating strategy for CD4+ T cell memory populations. (B) Summary dot plots showing the proportions of CD4+ T cells that are naive, central memory (CM), effector memory (EM), and terminally differentiated effector memory (TEMRA) cells. (C) Pie chart showing median percentages of CXCR5+ and CXCR5- CD4+ T cells. (D) Representative flow cytometry plots for CXCR5+ and CXCR5- gating within bulk CD4+ T cells and summary plots depicting the proportions of CXCR5+ and CXCR5- CD4+ T cells within the CM, EM, naive, and TEMRA subsets. Statistical analysis was done using the Kruskal-Wallis H test (B) and the Mann-Whitney U test (D).

controlling viral replication during primary HIV infection by enhancing robust anti-HIV antibody production, which is desirable for a prophylactic HIV vaccine.

(This article was submitted to an online preprint archive [18].)

**RESULTS**

**Circulating CXCR5+ cells in healthy donors have a predominantly central memory phenotype.** Recent studies have focused on characterizing circulating CXCR5+ CD4+ T follicular helper (cTfh) cells because of their similarities with germinal center Tfh cells and their potential role in the development of bNAbs (17, 19). The difficulty associated with obtaining bona fide Tfh cells from lymphoid tissues has also stirred the interest in studying cTfh cells as surrogates. Although the phenotype of cTfh cells has not been clearly defined, the consensus is that they represent circulating memory Tfh cells (13). To determine how HIV infection perturbs the global frequencies and phenotypes of peripheral Tfh cells, we began by establishing the baseline characteristics of this cell population in our study cohort, who were predominantly of Zulu/Xhosa ethnicity. We used CCR7 and CD45RA, well-established memory markers, to define four memory subsets. Specifically, we defined naive (N) T cells by gating on CCR7+ and CD45RA+ cells, central memory (CM) T cells by gating on CCR7+ CD45RA- cells, effector memory (EM) T cells by gating on CCR7- CD45RA- cells, and terminally differentiated effector memory (TEMRA) T cells by gating on CCR7- CD45RA+ cells (20) (Fig. 1A). Phenotypic analysis of total CD4+ T cells from 12 HIV-negative donors revealed that 34.0% (interquartile range [IQR], 29.1 to 43.2%) were naive, 21.8% (IQR, 19.1 to 28.0%) were CM, 33.7% (IQR, 30.4 to 44.4%) were EM, and 2.8% (IQR, 2.1 to 3.3%) were TEMRA (Fig. 1B). Next, we measured the frequency of cTfh (CXCR5+ CD4+) cells

**TABLE 1** Characteristics of study participants

Characteristic	Values(s) for:	
	HIV-negative participants	Participants with acute HIV infection
No. of participants	12	14
No. of males	0	6
No. of females	12	8
Median (IQR) CD4 count (no. of cells/ $\mu$ l)	NA <sup>a</sup>	463 (422–561)
Median (IQR) viral load (no. of copies/ml)	NA	121,000 (8,984–352,000)
Median (IQR) time postinfection (wk)	NA	6.9 (5.0–8.0)

<sup>a</sup>NA, not applicable.

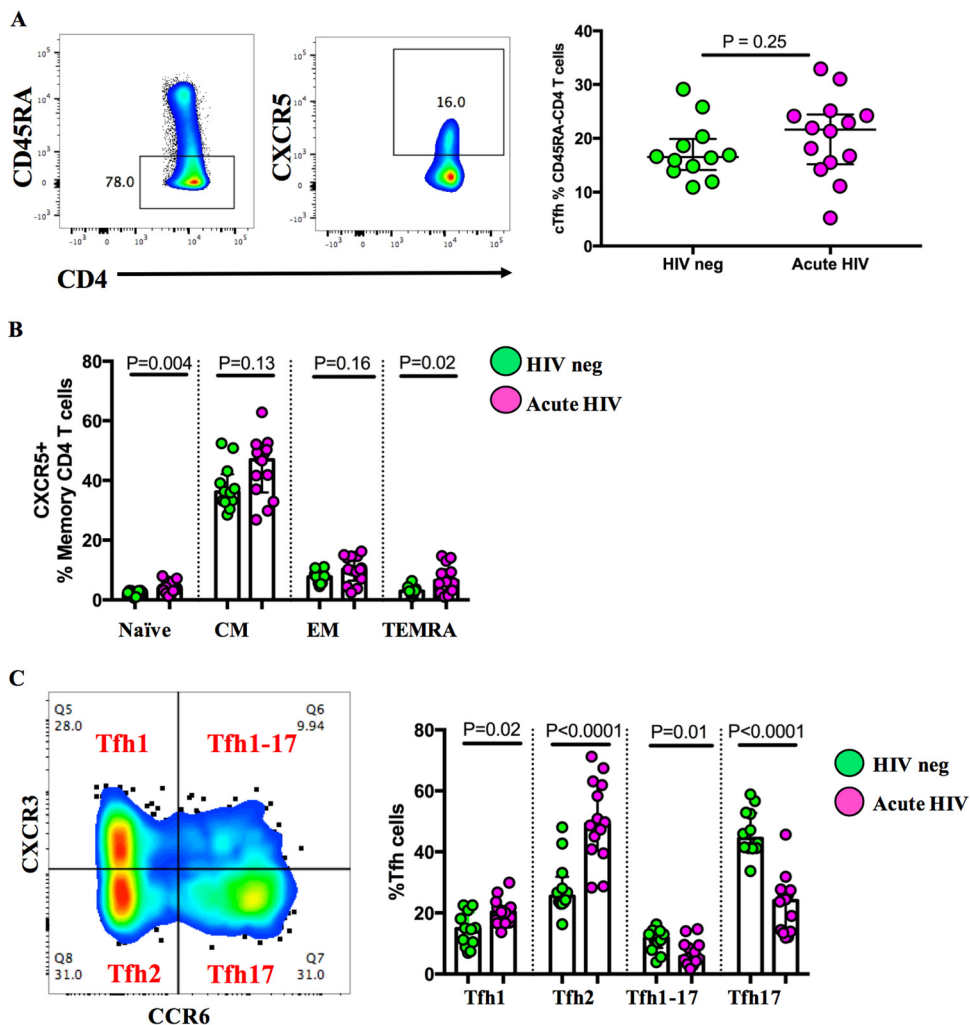
and found that they comprised 12% (IQR, 10.1 to 14.3%) of circulating CD4<sup>+</sup> T cells (Fig. 1C). Memory phenotyping of Tfh cells showed that cTfh cells comprised 37.3% of CM CD4<sup>+</sup> T cells, 7.8% of EM CD4<sup>+</sup> T cells, and only a paltry 2.6% and 2.9% of the naive and TEMRA CD4<sup>+</sup> T cell compartments, respectively (Fig. 1D). Consistent with studies in Caucasian populations (21, 22), our data show that cTfh cells constitute a small fraction of circulating CD4<sup>+</sup> T cells and are predominantly of a central memory phenotype.

**Perturbation of circulating Tfh cells during acute HIV-1 infection.** Having established the normal frequencies and phenotypes of circulating Tfh cells, we next investigated how acute HIV infection alters the frequency and differentiation profiles of these cells. Samples obtained at a median of 6.9 weeks after diagnosis of HIV infection were used for these studies (Table 1). As shown in Fig. 2A, HIV infection did not alter the overall frequencies of total circulating memory Tfh cells. However, memory subset analysis revealed an increase in naive Tfh ( $P = 0.004$ ) and TEMRA Tfh ( $P = 0.02$ ) cells, whereas CM Tfh ( $P = 0.13$ ) and EM Tfh ( $P = 0.16$ ) cells remained unchanged (Fig. 2B).

Next, we used CXCR3 and CCR6 chemokine receptor markers to characterize cTfh subsets in an effort to identify which subset most influences the generation of anti-HIV antibodies during acute HIV infection. CXCR3 and CCR6 chemokine receptor markers have been previously used to identify several functional subsets that exhibit distinct B cell helper functions, namely, the Tfh1 (CXCR3<sup>+</sup> CCR6<sup>-</sup>), Tfh2 (CXCR3<sup>-</sup> CCR6<sup>-</sup>), Tfh1-17 (CXCR3<sup>+</sup> CCR6<sup>+</sup>), and Tfh17 (CXCR3<sup>-</sup> CCR6<sup>+</sup>) subsets (13). A representative flow plot, as seen in Fig. 2C, depicts the distribution of cTfh subsets in an acutely infected donor based on the expression levels of the two respective chemokine receptor markers. Interestingly, acute infection skewed the distribution of cTfh subsets toward the Tfh1 ( $P = 0.02$ ) and Tfh2 ( $P < 0.0001$ ) phenotypes, with a significant reduction in the proportions of the Tfh1-17 ( $P = 0.01$ ) and Tfh17 ( $P < 0.0001$ ) phenotypes compared to those in HIV-negative donors being detected (Fig. 2C).

**Frequency of Tfh1 cells during early acute HIV-1 infection correlates negatively with SPVL.** Having observed a significant expansion of Tfh1 and Tfh2 cells, we next investigated if there was a relationship between the expanded cTfh subsets and set point viral load (SPVL), which is a reliable predictor of the rate of HIV disease progression. We calculated SPVL as the average viral load (VL) from 3 to 12 months postinfection, as previously reported (23), and correlated it to the frequencies of different cTfh subsets. Strikingly, Tfh1 cell frequencies correlated negatively with SPVL ( $P = 0.03$ , Spearman rho [ $r$ ] =  $-0.60$ ) (Fig. 3A), but there were no significant associations between Tfh2, Tfh1-17, Tfh17, or bulk cTfh cells and SPVL (Fig. 3B to E). These results suggest that Tfh1 cells contribute to viral control during the first year of infection.

**Tfh1 responses during early acute infection correlate with the p24 IgG responses detected at 1 year postinfection.** Numerous studies have associated slower disease progression with higher serum levels of HIV-1 Gag-specific IgG antibodies (reviewed in reference 24). We next hypothesized that Tfh1 responses impact SPVL by driving the production of HIV-specific IgG antibodies. We measured plasma gp41-, gp120-, p17-, and p24-specific IgG antibody titers at 12 months after infection for 10 study participants based on sample availability. Correlation analysis of IgG titers with SPVL revealed a negative correlation between SPVL and p24 IgG titers ( $P = 0.007$ ,  $r = -0.81$ )

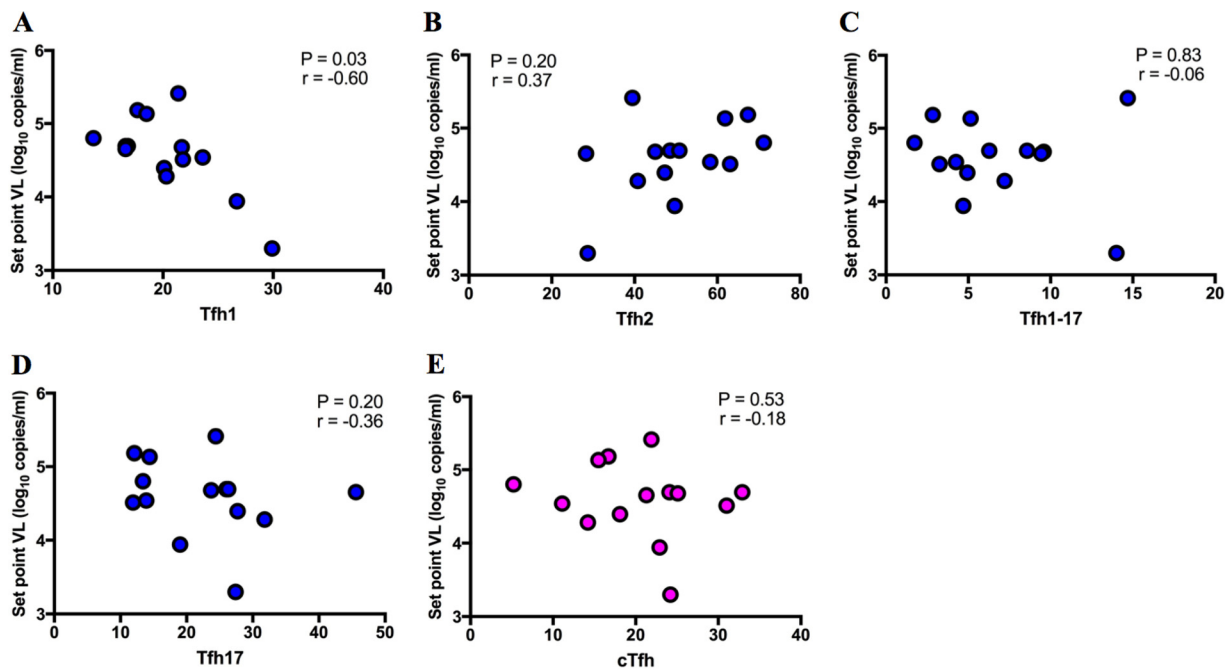


**FIG 2** Heterogeneity within the circulating Tfh compartment during acute HIV-1 infection. (A) Representative flow cytometry plots showing the gating strategy for bulk cTfh cells within CD45RA<sup>-</sup> CD4<sup>+</sup> T cells and summary proportions of cTfh cells in the HIV-negative (HIV neg) and acute HIV infection groups. (B) Summary plot comparing the frequencies of naive, CM, EM, and TEMRA cTfh cells in HIV-negative and acute HIV infection donors. (C) Gating strategy for the Tfh1, Tfh1-17, Tfh2, and Tfh17 subsets. The proportions of Tfh1, Tfh2, Tfh1-17, and Tfh17 subsets were compared between the HIV-negative and acute HIV infection groups. *P* values are from Mann-Whitney *U* tests.

(Fig. 4A) or gp41 IgG titers (*P* = 0.009, *r* = -0.80) (Fig. 4B) and no significant correlations between SPVL and p17 IgG titers (*P* = 0.09, *r* = -0.58) (Fig. 4C) or gp120 IgG titers (*P* = 0.20, *r* = -0.44) (Fig. 4D). We also examined the correlation of SPVL to the titers of p24 IgG isotypes IgG1, IgG2, IgG3, and IgG4 and found no significant correlations between the p24 IgG isotypes and SPVL (Fig. 4E and data not shown).

Lastly, we interrogated the relationship between Tfh1 frequencies and antibody titers. We found that Tfh1 frequencies during early infection (5.0 to 8.0 weeks) were directly correlated to the plasma titers of p24 IgG (*P* = 0.003, *r* = 0.85), p17 IgG (*P* = 0.01, *r* = 0.77), gp41 IgG (*P* = 0.05, *r* = 0.65), and p24 IgG1 (*P* = 0.04, *r* = 0.66) that were detected at 1 year postinfection (Fig. 4F to I). There was, however, no association between gp120 titers and Tfh1 frequencies (Fig. 4J). These results suggest that the polarization of cTfh responses toward a Tfh1 phenotype can potentially impact the development of long-lasting antibody responses.

**HIV-specific Tfh responses are induced during acute HIV-1 infection.** Next, we investigated if the expanded cTfh cell population in acute HIV infection was HIV specific using an intracellular cytokine staining (ICS) assay and major histocompatibility com-

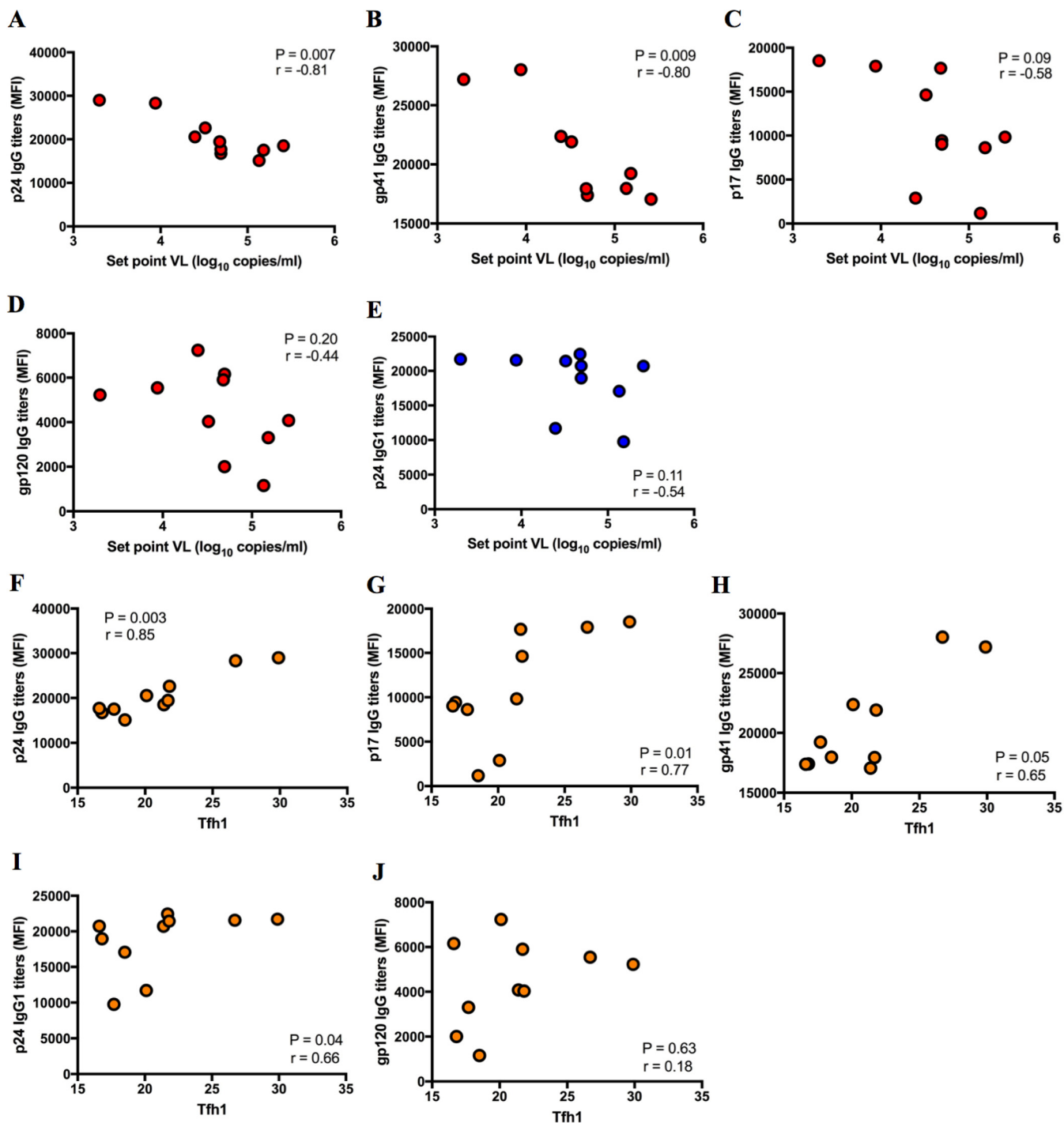


**FIG 3** Tfh1 correlates negatively with the set point viral load. The set point viral load was plotted against the frequency percentages of Tfh1 (A), Tfh2 (B), Tfh1-17 (C), Tfh17 (D), and bulk cTfh (E) cells determined by flow cytometry. Spearman rho (*r*) values and *P* values are reported.

plex (MHC) class II tetramers. Although HIV-specific CD4<sup>+</sup> T cells are important for viral control (25), the presence of HIV-specific Tfh responses in the circulation remains controversial (11, 17). Therefore, we interrogated the cytokine expression pattern of cTfh cells after stimulation with HIV peptides. Figure 5A shows representative flow plots of unstimulated controls (top) and cytokine-secreting antigen-specific CD4<sup>+</sup> cells responding to HIV peptide pools (middle) or staphylococcal enterotoxin B (SEB) stimulation (bottom) in an ICS assay. Our group previously showed that most of the HIV-specific CD4<sup>+</sup> T cells in chronic clade C infection target the HIV Gag protein (26). Here we found no significant differences in Gag, Nef, and Env responses (Fig. 5B). Further interrogation of the cytokine profile of cTfh cells revealed that, unlike SEB-specific cells, which abundantly secreted tumor necrosis factor alpha (TNF- $\alpha$ ) and gamma interferon (IFN- $\gamma$ ) (Fig. 5C), HIV-specific cTfh cells were biased toward the secretion of the Tfh cell functional cytokines IL-21 and IL-4, with lower proportions of cTfh cells secreting TNF- $\alpha$  and IFN- $\gamma$  (Fig. 5D). These differences, however, did not reach statistical significance after correcting for multiple comparisons (Fig. 5D). Comparative analysis with non-cTfh cells revealed that HIV-specific cTfh cells (blue) secreted more IL-21 (Fig. 5Ei, ii, and iv) and IL-4 (Fig. 5Eii and iii), whereas non-cTfh cells (red) secreted significantly more IFN- $\gamma$  (Fig. 5Ei and iv).

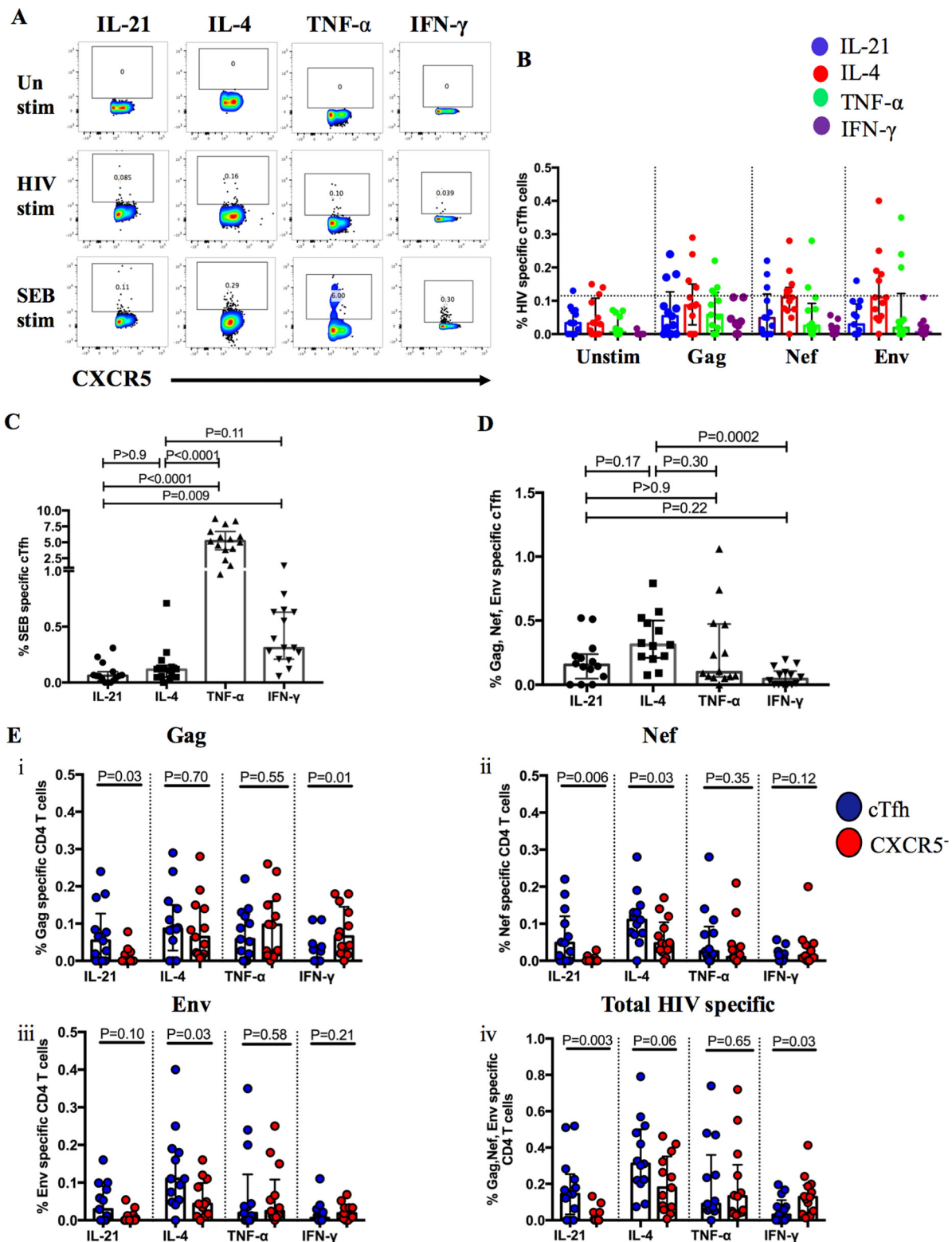
**Persistence of Gag-specific Tfh responses during HIV-1 infection.** We further used MHC class II tetramers to confirm the presence of HIV-specific cTfh subsets. Samples from seven HIV-infected participants (Table 2) expressing either the DRB1\*11:01 (*n* = 6) or the DRB1\*13:01 (*n* = 1) class II HLA haplotype were analyzed. As shown in Fig. 6A and B, dual-tetramer-positive (Tet<sup>++</sup>) CD4<sup>+</sup> T cells were detectable in HIV-infected patients but not in class II HLA-matched HIV-negative controls (*P* = 0.02). Further phenotypic analysis revealed an enrichment of tetramer-specific CXCR5<sup>-</sup> CD4<sup>+</sup> T cells compared to cTfh cells (*P* = 0.006) (Fig. 6C).

To define tetramer-specific cTfh subsets and to track their dynamics over time, we used longitudinal samples from one participant with acute HIV infection (patient 1) who had strong a response to the clade C Gag OLP 41 epitope restricted by the DRB1\*11:01 HLA haplotype. An overlay of the dual-tetramer-positive CD4<sup>+</sup> population onto CXCR5<sup>+</sup> CXCR3<sup>+</sup> CD4<sup>+</sup> cTfh cells showed that HIV-specific cTfh cells were predomi-



**FIG 4** Early Tfh1 responses are predictive of p24 IgG responses at 1 year of infection. (A to E) p24 IgG titers (A), gp41 IgG titers (B), p17 IgG titers (C), gp120 IgG titers (D), and p24 IgG1 titers (E) at the 1-year time point were determined using a customized multivariate Luminex assay, and the values were inversely correlated to SPVL. (F to J) p24 IgG (F), p17 IgG (G), gp41 IgG (H), p24 IgG1 (I), and gp120 IgG (J) titers at the 1-year time point were correlated to the frequencies of Tfh1 cells at 6.9 weeks (IQR, 5.0 to 8.0 weeks) of infection. MFI, mean fluorescence intensity; VL, viral load. Spearman rho (*r*) values and *P* values are reported.

nantly CXCR3<sup>+</sup> (Tfh1 and Tfh1-17) cells and were detectable at 12, 14, 16, and 20 weeks postinfection (Fig. 6D). This result was mirrored by that for another participant (patient 2) sampled at 6 weeks and 138 weeks postinfection (Fig. 6E). Combined data for the participants revealed significantly higher frequencies of Tfh1 and Tfh1-17 tetramer-specific cells than Tfh2 and Tfh17 tetramer-specific cells (*P* = 0.0007) (Fig. 6F). Together, these results demonstrate that HIV-specific cTfh cells persist during HIV infection.



**FIG 5** HIV-specific cTfh measurements using an ICS assay. (A) Representative flow cytometry plots for cytokine-secreting cTfh cells. PBMCs were unstimulated or stimulated with SEB or HIV OLP pools for Gag, Nef, and Env for 16 h in the presence of GolgiStop and GolgiPlug transport inhibitors (BD Biosciences), and the intracellular expression of IL-21, IL-4, TNF- $\alpha$ , and IFN- $\gamma$  was measured. (B) Summary frequency plots for unstimulated and Gag-, Nef-, and Env-specific cTfh cells (the horizontal line denotes the background threshold based on the responses under unstimulated control conditions). (C) Summary plots for SEB-stimulated cells. (D) Total HIV-specific cTfh cells. IL-21-, IL-4-, TNF- $\alpha$ -, and IFN- $\gamma$ -positive cTfh cells were summed for Gag, Nef, and Env. (E) Comparison of the cytokine secretion profiles of cTfh (CXCR5<sup>+</sup>) and non-cTfh (CXCR5<sup>-</sup>) cells. Frequencies for Gag (i), Nef (ii), and Env-specific (iii) cells were plotted separately or totaled (iv). *P* values are from Dunn's multiple-comparison test (C and D) and the Mann-Whitney U test (E).



**TABLE 2** Characteristics of study participants for tetramer staining assay

Characteristic	Values(s) for:		
	HIV-negative participants	Participants with acute HIV infection	Participants with chronic HIV infection
No. of participants	3	2	5
No. of males	0	0	0
No. of females	3	2	5
Median (IQR) CD4 count (no. of cells/ $\mu$ l)	NA <sup>a</sup>	365 (351–433)	720 (537–1,022)
Median (IQR) viral load (no. of copies/ml)	NA	18,760 (9,642–377,905)	2,950 (455–30,975)

<sup>a</sup>NA, not applicable.

## DISCUSSION

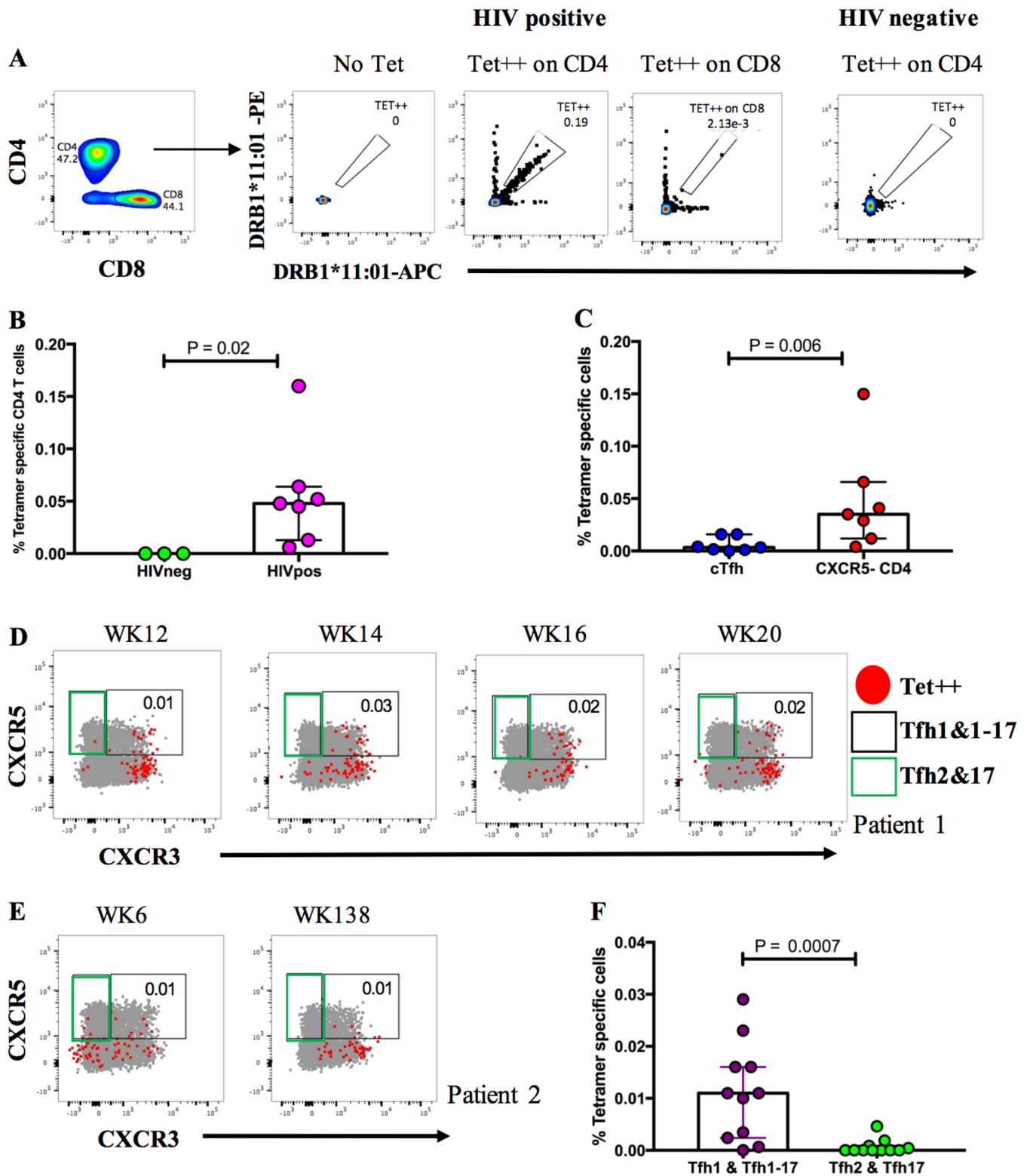
The extreme genetic diversity of HIV is a significant obstacle in the development of an effective anti-HIV vaccine (27). Even with the identification and isolation of several potent bNAbs in recent years, induction of such antibodies *in vivo* by vaccination has been a challenge (27, 28). Furthermore, nnAbs have been associated with protection from HIV acquisition and could be easier to induce by immunization than bNAbs (29). This study sheds new light on circulating CD4<sup>+</sup> T cell help that can impact the development of effective nonneutralizing anti-HIV antibody responses.

To understand how HIV modulates the frequency and function of circulating HIV-specific Tfh responses during primary HIV infection, we first established the baseline frequencies of cTfh cells in HIV-uninfected individuals. Comparative analysis between HIV-infected and -uninfected individuals showed that there are similar frequencies of total memory cTfh cells across both groups. A more in-depth phenotypic characterization of cTfh cells revealed four distinct functional subsets, namely, Tfh1, Tfh2, Tfh1-17, and Tfh17 cells. We next showed that the increased frequency of Tfh1 cells positively correlated with p24 IgG antibody responses and negatively correlated with the set point viral load. These data suggest that the Tfh1 subset plays an important role in the induction of anti-HIV antibodies and may contribute to the control of HIV replication, consistent with murine model studies which have shown that cTfh cells can traffic into lymph nodes and interact with B cells in interfollicular zones and in germinal centers (30).

The differential induction of cTfh subsets has been described in the context of other infectious diseases. Consistent with our data, the early induction of circulating CXCR3<sup>+</sup> cTfh cells, which comprise the Tfh1 and Tfh1-17 subsets, correlated with the emergence of protective responses to the influenza vaccine (31). In a subsequent study, the same investigators further demonstrated that CXCR3<sup>+</sup> Tfh cells promote the development of high-avidity antibody responses to the H1N1 vaccine (32). The aforementioned studies and our data suggest that Tfh1 cells might play an important helper role in the production of efficacious antiviral nnAbs. However, since studies using *in vitro* Tfh and B cell coculture assays have shown that CXCR3<sup>+</sup> Tfh cells are effective in providing help to memory B cells but deficient at offering naive B cell help (11, 17), more mechanistic work using animal models will be critical to delineating the intricacies of circulating Tfh1 cell helper capacity and providing clarity on the functional ability of Tfh1 subsets.

From our results, we also observed an expansion of the Tfh2 subsets during acute HIV-1 infection compared to that in the controls. The CXCR3<sup>-</sup> subset, which comprises the Tfh2 and Tfh17 subsets, has been described as having a superior helper capacity *in vitro* (11, 17), and the frequencies during acute HIV-1 infection were predictive of the ability to develop bNAbs in one study (17). However, another study did not see any relationship between this subset and the ability to develop bNAbs (22). Although we sought to determine the relationship between the Tfh2 subset and bNAb development in our study, only one study participant developed bNAbs; thus, we were unable to make any conclusions.

Several reports have implicated bulk CD4<sup>+</sup> T cells in immune-mediated control of chronic HIV infection (25, 26, 33), but little is known about the role of HIV-specific cTfh



**FIG 6** HIV-specific cTfh cell detection by HLA class II tetramers. (A) Representative flow cytometry plots showing the gating strategy for dual-tetramer-positive (Tet<sup>++</sup>) cells within CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells. (B) Frequencies of Tet<sup>++</sup> CD4<sup>+</sup> T cells for HIV-negative and HIV-infected donors. (C) Percentages of Tet<sup>++</sup> cTfh cells and Tet<sup>++</sup> CXCR5<sup>-</sup> cells within CD4<sup>+</sup> T cells. (D and E) Overlay plots showing Tet<sup>++</sup> cells (red dots) within cTfh subsets. (D and E) HIV-specific cTfh cells were detected at 12, 14, 16, and 20 weeks (D) or at 6 and 138 weeks (E) postinfection. Black, CXCR3<sup>+</sup> CXCR5<sup>+</sup> gate; green, CXCR3<sup>-</sup> CXCR5<sup>+</sup> gate. (F) Summary plots showing the frequencies of tetramer-specific CXCR3<sup>+</sup> (Tfh1 and Tfh1-17) and CXCR3<sup>-</sup> (Tfh2 and Tfh17) cTfh cells. P values are from the Mann-Whitney U test.

cells in HIV control, mainly because of their very low frequency in the circulation and the paucity of reliable tools to study them. Even though there were low numbers of cytokine-secreting cTfh cells in response to stimulation by HIV peptide, as previously shown (34), our tetramer staining results provided conclusive evidence of the existence of HIV-specific cTfh cells during primary HIV infection. Notably, unlike bulk HIV-specific CD4<sup>+</sup> T cells, which mostly target Gag, our data show that cTfh responses during acute HIV infection are dynamic and comprise a broad repertoire of cells specific for the HIV-1 Gag, Nef, and Env proteins. Virus-specific cTfh cells targeting different HIV proteins may have a synergistic antiviral effect via cross talk through the so-called intrastructural help to promote a greater net antiviral effect. This concept was first demonstrated in simian immunodeficiency virus MAC (SIV<sub>MAC</sub>) Gag adenoviral vector-immunized macaques and later validated by a murine model of SIV<sub>MAC</sub> infection (35, 36). In the initial study, a faster onset and a stronger magnitude of antibody-dependent cell-mediated virus inhibition mediated by Env-specific antibodies were observed in immunized animals than in the controls (35, 36). Human studies of cTfh cells comparing the effector profile of cTfh cells having different HIV protein specificity showed that Env-specific cTfh cells were superior at inducing class switching to IgG, while Gag-specific cTfh cells were better at inducing B cell proliferation and maturation (37). These assays were conducted *in vitro*, but the microanatomy of immune responses *in vivo* might encourage interactions between cells of different specificities. Additionally, studies have alluded to some degree of promiscuity in Tfh cell help to B cells in the GCs. It has been shown that the Tfh response is polyclonal (38); also, the egression of Tfh cells from their initially colonized GCs and migration into other GCs have been documented (38, 39). These kinds of results argue for a less rigid Tfh cell help and highlight the dynamism of Tfh cell-B cell interactions, which are the subject of many studies.

As mentioned above, our tetramer staining results give a strong indication that cTfh cells persist in the circulation well into chronic HIV infection. Although there were significantly higher frequencies of Tfh2 cells than Tfh1 cells during acute HIV infection, there was a higher proportion of tetramer-specific Tfh1 cells. The tetramers that we tested were directed at the Gag C41 epitope, and one possibility is that Tfh2 cells may be targeting an epitope other than the Gag C41 epitope, which we interrogated. We, however, consider the expansion of the Tfh2 subset to be an interesting observation that warrants further studies.

Our data reveal important associations between nnAbs and the SPVL. The exact mechanism of how nnAbs influence HIV replication requires further investigation. Nevertheless, we speculate that the negative correlation between antibody titers and the lower SPVL may be attributable to antibody effector functions that have been associated with improved virus control (40) and slower HIV disease progression (41, 42). Fc effector functions, like antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), have been described to be important for virus control, and these functions are mostly attributed to Env-specific IgG antibodies (41, 42). Additionally, studies investigating the mechanisms for virus suppression by Gag-specific antibodies have described the ability of Gag-specific antibodies to opsonize antigens and recruit conventional or plasmacytoid dendritic cells to phagocytose antibody-coated antigens (43–45). These opsonophagocytic IgG responses were associated with lower plasma HIV RNA levels (43, 46), thus highlighting another potential mechanism of virus control. Interestingly, Gag p24 antibodies and gp41 antibodies independently correlated with the SPVL, whereas gp120-specific antibodies did not. The reason that gp41 but not gp120 IgG antibodies correlate with the SPVL remains an open question, but it could be because functional epitopes for Fc binding antibodies reside in gp41. Alternatively, it could be due to the reported differences in the kinetics of the two antibody specificities (47, 48).

Early studies investigating the kinetics and magnitude of anti-Gag and anti-Env IgG antibodies observed that the decay of Gag-specific antibodies correlated with poorer disease outcomes and argued that Gag-specific antibodies are a surrogate for CD4 T cell help to Gag-specific CD8 T cells (49). CD8 T cells are important for virus control, and

robust IL-21-mediated Tfh cell help to CD8 T cells improves CD8 T cell cytolytic activity (37), but we observed no correlations between the frequencies of IFN- $\gamma$ -positive CD8 T cells and lower set point viral loads among our study participants. Additionally, a paper from our group showed that the association between Gag p24 IgG and viral control was still maintained even after controlling for Gag-specific CD4 and CD8 T cell responses, suggesting a CD8 T cell-independent antiviral mechanism of these antibodies (50).

A notable limitation of the study is the small sample size, due to difficulty in recruiting subjects with untreated acute HIV-1 infection in the present era of mass antiretroviral therapy (ART) induction in all HIV-1-infected patients. Nevertheless, despite the small sample size, we generated statistically significant results that provide new insight into the role of cTfh cells and their impact on the induction of antibody responses during primary HIV infection. Further studies to validate our findings in other acute infection cohorts are warranted.

In conclusion, the present study has identified a circulating Tfh1 subset whose frequency during acute HIV infection predicts the development of anti-p24 nonneutralizing antibodies. We also show that higher p24 IgG titers contribute to the control of HIV replication and have a beneficial effect on HIV disease progression. These results highlight the important role of HIV-specific cTfh cells in the generation of robust anti-HIV antibody responses, which are desirable for an HIV vaccine. Additionally, the identification of a cTfh subset that predicts the development of highly functional antibody responses might be useful to vaccine trials/studies as a potential biomarker to predict the development of robust antibody responses in vaccine responders or as a potential cell subset that can be manipulated to enhance vaccine responses (17).

## MATERIALS AND METHODS

**Study participants.** Study participants comprised 16 individuals with acute HIV infection and 5 chronically HIV-infected ART-naive individuals from the HIV Pathogenesis Programme (HPP) Acute Infection Cohort, Durban, South Africa. Patients were chosen based on the availability of acute infection samples. Acute infection classification and disease staging in this cohort were previously described (23). Briefly, at screening, patients had detectable HIV RNA but had not yet seroconverted either by enzyme-linked immunosorbent assay or by Western blotting. The date of infection for the study participants was estimated to be 14 days prior to screening, as previously described (51). One acute infection time point was selected per patient for the study based on sample availability. The time postinfection across the patients was a median of 7 weeks (IQR, 5.25 to 7.75 weeks). The CD4 count, viral load, and other patient characteristics are summarized in Table 1.

Fifteen HIV-uninfected individuals from the Females Rising through Education, Support, and Health (FRESH) cohort (52, 53), also in Durban, South Africa, were included as controls. The controls were chosen randomly based on sample availability at the time that the study was conducted. The University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) and the Massachusetts General Hospital Ethics Review Board approved the study. All study participants signed informed consent for participation in the study.

**Immunophenotyping.** For surface phenotyping, frozen peripheral blood mononuclear cells (PBMCs) were thawed, rested, and stained using a LIVE/DEAD Aqua dead cell staining kit (Thermo Fisher Scientific, Waltham MA, USA) per the manufacturer's instructions, followed by staining with an antibody panel comprising CD14 Horizon Violet 500 (HV500) (BD Biosciences, San Jose, CA), CD19 HV500 (BD Biosciences), CD3 Brilliant Violet 711 (BV711) (BioLegend, San Diego, CA, USA), CD8 Qdot 800 (Life Technologies, Carlsbad, CA, USA), CD4 Qdot 655 (Life Technologies), CXCR5 Alexa Fluor 488 (AF488) (BD Biosciences), PD-1 BV421 (BioLegend), CCR6 phycoerythrin (PE) (BioLegend), CXCR3 BV605 (BioLegend), CD45RA PE-cyanine 7 (PE-Cy7) (BioLegend), CCR7 peridinin chlorophyll protein-Cy5.5 (BioLegend), and CD27 allophycocyanin-Hilite 7 (APC-H7) (BD Biosciences). For intracellular cytokine staining, peripheral blood mononuclear cells (PBMCs) were either left unstimulated or stimulated with HIV clade C overlapping peptide (OLP) pools spanning the Gag, Nef, or Env protein or staphylococcal enterotoxin B (SEB; 0.5  $\mu$ g/ml) in the presence of GolgiStop and GolgiPlug protein transport inhibitors (BD Biosciences) for 16 h at 37°C. Cells were surface stained, washed, fixed, and permeabilized using a BD Cytotfix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Cells were subsequently stained intracellularly with IL-2 PE (BD Biosciences), IL-21 allophycocyanin (APC) (BioLegend), TNF- $\alpha$  AF700 (BD Biosciences), IL-4 BV605 (BioLegend), and IFN- $\gamma$  PE-Cy7 (BioLegend) antibodies. Cells were acquired using an LSRFortessa cytometer (BD Biosciences) with FACSDiva software, and fluorescence-minus-one controls were used to define the gates for the different cell subsets. Data were analyzed using FlowJo (version 10.0.8) software (FlowJo, LLC).

**HLA class II tetramer staining.** HIV-specific cTfh responses were measured using HLA class II tetramers. The immunodominant Gag overlapping peptide (OLP) 41 epitope (26) was interrogated using DRB1\*11:01 and DRB1\*13:01 tetramers produced in the laboratory of Søren Buus as previously described (54). The design and validation of these tetramers by our group have also been described (26). Briefly,

recombinant human DRB1\*11:01 or DRB1\*13:01 HLA molecules were complexed with a clade C HIV-1 Gag OLP 41 (YVDRFFKTLRAEQATQDV). For the assay, PBMCs were stained for 1 h at 37°C with APC- and PE-conjugated HLA class II tetramer complexes, washed in 2% fetal calf serum (FCS) in phosphate-buffered saline (PBS), and then stained with the following antibodies for 20 min at room temperature: a LIVE/DEAD Fixable Blue dead cell stain kit (Thermo Fisher Scientific), CD3 BV711 (BioLegend), CD4 BV650 (BD Biosciences), CD8 BV786 (BD Biosciences), CXCR5 AF488 (BD Biosciences), CXCR3 BV605 (BioLegend), PD-1 BV421 (BioLegend), and CD45RA Alexa Fluor 700 (AF700) (BioLegend). Cells were washed and acquired on the LSRFortessa cytometer (BD Biosciences).

**Customized multivariate Luminex assay.** Plasma HIV-1-specific antibodies were measured using a customized multivariate Luminex assay as previously described (55). Carboxylated fluorescent polystyrene beads (Bio-Rad, Hercules, CA, USA) were coated with HIV-1-specific proteins, including gp120 of clade C strain ZA.1197MB, gp41 of clade C strain ZA.1197MB, C-terminal 6×His-tagged p24 subtype C, and p17 HXBc2 (Immune Technology, New York, NY, USA). Plasma samples were incubated with antigen-coated beads in a 96-well plate, and unbound antibodies were washed with 0.05% Tween 20 in PBS. HIV-1-specific IgG antibodies were detected with PE mouse IgG1, IgG2, IgG3, and IgG4 secondary antibodies.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism (version 7.0) software (GraphPad Software, La Jolla, CA, USA). Statistical significance was assessed using Mann-Whitney U tests and the Kruskal-Wallis H test or two-way analysis of variance with Dunn's multiple-comparison test. The correlations between two variables were done using Spearman's rank correlation. *P* values were considered significant if they were less than 0.05.

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