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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Running title: Circulating follicular helper $CD4^+$ T cell responses

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Keywords: HIV, T follicular helper cells, non-neutralizing antibodies, Gag p24 IgG

(Word count: 4173)

Abstract (Word count:243)

Despite decades of focused research, the field has yet to develop a prophylactic vaccine. In the RV144 vaccine trial, non-neutralizing 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) cell subsets to the development of non-neutralizing 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 characterization of cTfh were performed using HLA class II tetramers and intracellular cytokine staining. In this study, we found that acute HIV-1 clade C infection skewed differentiation of functional cTfh subsets towards increased Tfh1 ($p=0.02$) and Tfh2 ($p<0.0001$) subsets, with a concomitant decrease in overall Tfh1-17 (that shares both Tfh1 and Tfh17 properties) ($p=0.01$) and Tfh17 subsets ($p<0.0001$) compared to HIV negative subjects. Interestingly, the frequencies of Tfh1 during acute infection (5.0-8.0 weeks post-infection) correlated negatively with set point viral load ($p=0.03$, $r=-60$) and were predictive of p24-specific plasma IgG titers at one year of infection ($p=0.003$, $r=0.85$). Taken together, our results suggest that circulating the 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 (Word count:132)

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 lower set point viral load. This study highlights a key Tfh cell subset that could limit HIV replication by enhancing antibody generation. This study underscores the importance of circulating Tfh cells in promoting non-neutralizing antibodies during HIV-1 infection.

Introduction

A safe and effective prophylactic vaccine remains the most efficient way of ending the HIV/AIDS epidemic which affects over 36 million people worldwide (1). Although studies in non-human 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 $CD4^+$ 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 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 IL-4 like Th2 cells, Tfh17 cells express CCR6 similar to Th17 cells and Tfh1-17 cells have functional properties that are similar to both Th1 and Th17 cells (12-14).

From the RV144 vaccine trial, which had a modest efficacy in preventing HIV acquisition, we learned that non-neutralizing antibodies (nnAbs) could protect against HIV acquisition (15). 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 HIV (SHIV) in macaques and reduce the number of transmitted/founder viruses establishing infection in the animals (16). Moreover, a humanized mouse model of HIV infection, reported near-complete clearance of adoptively transferred infected cells within 5 hours of nnAbs treatment (17) 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 $CXCR3^-$ cTfh; which includes both Tfh2 and Tfh17 subsets, with the development of bNAbs against HIV infection (18), 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 specific Tfh subsets that contribute to nnAbs development in the context of natural HIV infection.

Here we investigated if the induction of cTfh responses during acute HIV infection contribute to initial HIV control and promote the development of anti-HIV nnAbs. We examined the role of HIV-specific cTfh cell 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 significant expansion of HIV-specific memory Tfh1 cells ($p=0.02$), which correlated with lower set point viral loads. 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 controlling viral replication during primary HIV infection by enhancing robust anti-HIV antibody production, which is desirable for a prospective HIV vaccine.

Results

Circulating $CXCR5^+$ cells in healthy donors have a predominantly central memory phenotype. Recent studies have focused on characterizing circulating $CXCR5^+CD4^+$ T cells (cTfh) because of their similarities with germinal center Tfh cells and their potential role in the

development of bNAbs (18, 19). The difficulty associated with obtaining *bona fide* Tfh from lymphoid tissues has also stirred the interest in studying cTfh as surrogates. Although the phenotype of cTfh has not been clearly defined, the consensus is that they represent circulating memory Tfh (13). To determine how HIV infection perturbs global frequencies and phenotypes of peripheral Tfh we began by establishing baseline characteristics of this cell population in our study cohort who are predominantly of the Zulu/Xhosa ethnicity. We used CCR7 and CD45RA, well established memory markers to define four memory subsets. Specifically, we defined naïve (N) T cells by gating on $CCR7^+$ and $CD45RA^+$, central memory (CM) by $CCR7^+CD45RA^-$, effector memory (EM) by $CCR7^-CD45RA^-$, and terminally differentiated effector memory (TEMRA) by $CCR7^-CD45RA^+$ (20) (Figure 1A). Phenotypic analysis of total $CD4^+$ T cells from 12 HIV negative donors revealed that 34.0% (29.1-43.2) were naïve, 21.8% (19.1-28.0) were CM, 33.7% (30.4-44.4) were EM and 2.8% (2.1-3.3) were TEMRA (Figure 1B). Next, we measured the frequency of cTfh ($CXCR5^+CD4^+$) cells and found that they comprised 12% (10.1-14.3) of circulating $CD4^+$ T cells (Figure 1C). Memory phenotyping of Tfh cells showed that cTfh cells comprised 37.3% of CM $CD4^+$ T cells, 7.8 % of EM $CD4^+$ T cells and only a paltry 2.6% and 2.9% of the naïve and TEMRA $CD4^+$ T cell compartments respectively (Figure 1D). Consistent with studies in Caucasian populations (21, 22), our data show that cTfh constitute a small fraction of circulating $CD4^+$ 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 HIV diagnosis were used for these studies (Table 1). As shown in

(Figure 2A), HIV infection did not alter the overall frequencies of total circulating memory Tfh. However, memory subset analysis revealed an increase in naïve Tfh (p=0.004) and TEMRA Tfh (p=0.02), whereas CM Tfh (p=0.13) and EM Tfh (p=0.16) remained unchanged (Figure 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: Tfh1 ($CXCR3^{+}CCR6^{-}$), Tfh2 ($CXCR3^{-}CCR6^{-}$), Tfh1-17 ($CXCR3^{+}CCR6^{+}$) and Tfh17 ($CXCR3^{-}CCR6^{+}$) (13). A representative flow plot, as seen in Figure 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 towards the Tfh1 (p=0.02) and Tfh2 (p<0.0001) phenotypes with significant reduction in the proportions of Tfh1-17 (p=0.01) and Tfh17 (p<0.0001) compared to HIV negative donors (Figure 2C).

Frequency of Tfh1 cells during early acute HIV-1 infection correlates negatively with set point viral load. Having observed a significant expansion of Tfh1 and Tfh2, 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 VL from 3 to 12 months' post-infection as previously reported (23) and correlated it to the frequencies of different cTfh subsets. Strikingly, Tfh1 frequencies correlated negatively with SPVL (p=0.03, r=-0.60) (Figure 3A) but there were no significant associations

between Tfh2, Tfh1-17, Tfh17 or bulk cTfh cells and SPVL (Figures 3B, 3C, 3D and 3E). These results suggest that Tfh1 cells contribute to viral control during the first year of infection.

Tfh1 responses during early acute infection correlate with p24 IgG responses detected at one year post-infection. Numerous studies have associated slower disease progression with higher serum levels of HIV-1 Gag-specific IgG antibodies [reviewed in (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 ($p=0.007$ $r=-0.81$) (Figure 4A) or gp41 IgG ($p=0.009$, $r=-0.80$) (Figure 4B) and no significant correlations between SPVL and p17 IgG ($p=0.09$, $r=-0.58$) (Figure 4C) or gp120 IgG titers ($p=0.20$, $r=-0.44$) (Figure 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 (Figure 4E and data not shown).

Lastly, we interrogated the relationship between Tfh1 frequencies and antibody titers. We found that Tfh1 frequencies during early infection (5.0-8.0 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 post-infection (Figures 4F, 4G, 4H and 4I). There was however, no association between gp120 titers and Tfh1 frequencies (Figures 4J). These results suggest that the polarization of cTfh responses towards 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 in acute HIV infection were HIV-specific using intracellular cytokine staining (ICS) assay and MHC class II tetramers. Although HIV-specific $CD4^+$ T cells are important for viral control (25), the presence of HIV-specific Tfh responses in circulation remains controversial (11, 18). 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 panel), cytokine secreting antigen specific $CD4^+$ cells responding to HIV peptide pools (middle panel) or SEB stimulation (bottom panel) in an ICS assay. Our group previously showed that most of the HIV-specific $CD4^+$ 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 (Figure 5B). Further interrogation of the cytokine profile of cTfh cells revealed that unlike SEB-specific cells which abundantly secreted TNF-α and IFN-γ (Figure 5C), HIV-specific cTfh were biased towards the secretion of Tfh functional cytokines: IL-21 and IL-4, with lower proportions of cTfh cells secreting TNF-α and IFN-γ (Figure 5D). Comparative analysis with non cTfh cells revealed that HIV-specific cTfh cells (blue) secreted more IL-21 (Figure 5E i, ii & iv) and IL-4 (Figure 5E ii & iii) whereas non cTfh cells (red) secreted significantly more IFN-γ (Figure 5E i & 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 and to track their dynamics over time. Using longitudinal samples for one acute HIV participant (patient 1) who had strong a response to the Gag C41 epitope restricted by DRB1*11:01 HLA haplotype, dual

tetramer positive cells were detected within $CD4^+$ T cells (Figure 6A). An overlay of these double tetramer $CD4^+$ population onto $CXCR5^+CXCR3^+CD4^+$ cTfh showed that HIV-specific cTfh were predominantly $CXCR3^+$ (Tfh1 and Tfh1-17) cells and were detectable at 12, 14, 16 and 20 weeks post-infection (Figure 6B). Persistent HIV-specific cTfh cells during HIV-1 infection, was observed in another acute patient (patient 2) at 6 weeks and 138 weeks post infection (Figure 6C). Five additional patients expressing either the DRB1*11:01 (n=4) or the DRB1*13:01 (n=1) HLA haplotypes had detectable HIV-specific cTfh responses in chronic infection (>2 years of infection). Combined data for the 7 patients tested (Table 2) showed significantly higher frequencies of $CXCR3^+$ (Tfh1 and Tfh1-17) tetramer-specific cells compared to Tfh2 and Tfh17 ($p=0.0007$) (Figure 6D). Together, these results demonstrate that HIV-specific cTfh cells persist during HIV infection.

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 as compared to bNAbs (29). This study sheds new light on circulating $CD4^+$ T cell help that can impact the development of effective non-neutralizing 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 baseline frequencies of cTfh cells in

HIV uninfected individuals. Comparative analysis between HIV infected and uninfected individuals showed there are similar frequencies of total memory cTfh cells across both groups. 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 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 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 $CXCR3^{+}$ cTfh, which comprises Tfh1 and Tfh1-17 subsets, correlated with the emergence of protective responses to the Influenza vaccine (31). In a subsequent study, they further demonstrated that $CXCR3^{+}$ 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 co-culture assays, have shown that $CXCR3^{+}$ Tfh cells are effective in providing help to memory B cells but deficient at offering naïve B cell help (11, 18), 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 the controls. The $CXCR3^-$ subset which comprises Tfh2 and Tfh17 subsets has been described as having superior helper capacity *in-vitro* (11, 18) and the frequencies during acute HIV-1 infection was predictive of the ability to develop bNAbs in one study (18). 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 bNAbs development in our study, only one study participant developed bNAbs thus, we were unable to make any conclusions.

Several reports have implicated bulk $CD4^+$ T cells in immune mediated control of chronic HIV infection (25, 26, 33), but little is known about the role of HIV-specific cTfh cells in HIV control mainly because of their very low frequency in circulation and the paucity of reliable tools to study them. Even though there were few 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 $CD4^+$ 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 HIV-1 Gag, Nef and Env proteins. Virus-specific cTfh cells targeting different HIV proteins may have 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 SIV_{MAC} Gag adenoviral vector immunized macaques and later validated by a murine model of SIV_{MAC} infection (35, 36). In the initial study, a faster onset and magnitude of antibody-dependent cell-mediated virus inhibition mediated by Env-specific antibodies, was observed in immunized

273 animals compared to controls (35, 36). Human studies of cTfh cells comparing the effector
274 profile of cTfh cells having different HIV-protein specificity, showed that Env-specific cTfh
275 cells were superior at inducing class switching to IgG while Gag-specific cTfh cells were better
276 at inducing B cell proliferation and maturation (37). These assays were conducted *in-vitro* but
277 the micro-anatomy of immune responses *in-vivo* might encourage interactions between cells of
278 different specificities. Additionally, studies have alluded to some degree of promiscuity in Tfh
279 cell help to B cells in the GCs. It has been shown that the Tfh response is polyclonal (38), also
280 the egression of Tfh cells from their initial colonized GCs and migration into other GCs have
281 been documented (38, 39). These kinds of results argue for a less rigid Tfh help and highlight the
282 dynamism of Tfh cell-B cell interactions which are the subject of many studies.
283
284 As previously mentioned, our tetramer staining results give a strong indication that cTfh cells
285 persist in circulation well into chronic HIV infection. Although there were significantly higher
286 frequencies of Tfh2 cells compared to Tfh1 cells during acute HIV, there was a higher proportion
287 of tetramer specific Tfh1 cells. The tetramers we tested were directed at the Gag C41 epitope and
288 one possibility is that Tfh2 cells may be targeting a different epitope other than the Gag C41
289 epitope which we interrogated. We however, consider the expansion of the Tfh2 subset as an
290 interesting observation that warrants further studies.
291
292 Our data reveals an important association between Gag p24-specific nnAbs and viral load set
293 point. The exact mechanism of how nnAbs influence HIV replication requires further
294 investigation. Nevertheless, we speculate that the negative correlation between antibody titers
295 and lower viral load set point may be attributable to antibody effector functions that have been

296 associated with improved virus control (40) and slower HIV disease progression (41, 42).
297 Although Fc effector functions like antibody-dependent cellular cytotoxicity (ADCC) and
298 antibody-dependent cellular phagocytosis (ADCP) have been described as important for virus
299 control, most of these functions were mostly attributed to Env-specific IgG antibodies. (41, 42).
300 Studies investigating the mechanisms for virus suppression by Gag-specific antibodies have
301 described the ability of these Gag-specific antibodies to opsonize antigens and recruit
302 conventional or plasmacytoid dendritic cells to phagocytose antibody-coated antigens (43-45).
303 Additionally, these opsonophagocytic IgG responses were associated with lower plasma HIV-
304 RNA levels (43, 46), thus, highlighting this as the potential mechanism of virus control.
305 Nevertheless, investigations into these and other antibody effector mechanisms are underway for
306 this cohort.
307
308 Early studies investigating the kinetics and magnitude of anti-Gag and anti-Env IgG antibodies
309 observed that the decay of Gag-specific antibodies correlated with poorer disease outcomes and
310 argued that Gag-specific antibodies are a surrogate for CD4 T cell help to Gag-specific CD8 T
311 cells (47). CD8 T cells are important for virus control and robust IL-21 mediated Tfh help to
312 CD8 T cells improves CD8 T cell cytolytic activity (37), but we observed no correlations
313 between the frequencies of IFN-γ^+ CD8 T cells and lower set point viral loads among our study
314 participants. Additionally, a paper from our group now in press showed that the association
315 between Gag-p24 IgG and viral control was still maintained even after controlling for Gag-
316 specific CD4 and CD8 T cell responses suggesting a CD8 T cell independent anti-viral
317 mechanism of these antibodies (48).
318

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 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 non-neutralizing 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 bio-marker 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 (49).

Materials and Methods

Study Participants

Study participants comprised of 16 acute and 5 chronic HIV-infected ART-naïve individuals from HIV Pathogenesis Programme (HPP) Acute Infection cohort, Durban, South Africa. Patients were chosen based on availability of acute infection samples. Acute infection classification and disease staging in this cohort was previously described (23). Briefly, at

342 screening, patients had detectable HIV RNA but had not yet seroconverted, either by ELISA or
343 Western blotting. The date of infection for the study participants was estimated to be 14 days
344 prior to screening as previously described (50). One acute infection time point was selected per
345 patient for the study based on sample availability. The time post-infection across the patients was
346 a median of 7 weeks (interquartile range-IQR, 5.25-7.75). The CD4 count, viral load and other
347 patient characteristics are summarized in table 1.
348 10 HIV uninfected individuals from the Females Rising Through Education, Support and Health
349 (FRESH) cohort (51, 52), also in Durban, South Africa were included as controls. The controls
350 were chosen randomly based on sample availability at the time the study was conducted. The
351 University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) and the
352 Massachusetts General Hospital ethics review board approved the study. All study participants
353 signed informed consent for participation in the study.
354
355 **Immunophenotyping**
356 For surface phenotyping, frozen PBMCs were thawed, rested and stained using the LIVE/DEAD
357 Aqua dead cell staining kit (Thermofisher scientific, Waltham MA, USA) as per manufacturer's
358 instructions, followed by staining with an antibody panel comprising: CD14 HV500 (BD
359 Biosciences, San Jose, CA), CD19 HV500 (BD Biosciences), CD3 BV711 (BioLegend, San
360 Diego, CA, USA), CD8 Qdot 800 (Life Technologies, Carlsbad, CA, USA), CD4 Qdot 655 (Life
361 Technologies), CXCR5 AF488 (BD Biosciences), PD-1 BV421 (BioLegend), CCR6 PE
362 (BioLegend), CXCR3 BV605 (BioLegend), CD45RA PE-Cy7 (BioLegend), CCR7 PerCp Cy5.5
363 (BioLegend) and CD27 APCH7 (BD Biosciences). For intracellular cytokine staining, peripheral
364 blood mononuclear cells (PBMCs) were either left unstimulated or stimulated with HIV clade C

overlapping peptide (OLP) pools spanning Gag, Nef, or Env proteins or staphylococcal enterotoxin B (SEB, 0.5 μg/ml) in the presence of GolgiStop and GolgiPlug protein transport inhibitors (BD Biosciences) for 16 hours at 37°C. Cells were surface stained, washed, fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer's instructions. Cells were subsequently stained intracellularly with IL-2 PE (BD Biosciences), IL-21 APC (BioLegend), TNF-α A700 (BD Biosciences), IL-4 BV605 (BioLegend) and IFN-γ 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 gates for the different cell subsets. Data was analysed using the FlowJo version 10.0.8 (Flowjo, LLC).

HLA class II tetramer staining

HIV-specific cTfh responses were measured using HLA class II tetramers. The immunodominant Gag C41 epitope (26) was interrogated using DRB1*11:01 and DRB1*13:01 tetramers produced in the laboratory of Dr Søren Buus as previously described (53). 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 clade C HIV-1 Gag 41 peptide (YVDRFFKTLRAEQATQDV). For the assay, PBMCs were stained for 1 hour at 37^{o}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 these antibodies: LIVE/DEAD Fixable Blue dead cell stain kit (Thermofisher 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 AF700 (BioLegend); for

20 min at room temperature. Cells were washed and acquired on the LSRFortessa (BD Biosciences).

Customized multivariate Luminex assay

Plasma HIV-1 specific antibodies were measured using a customized multivariate Luminex assay as previously described (54). Carboxylated fluorescent polystyrene beads (Biorad, Hercules, CA, USA) were coated with HIV-1 specific proteins including gp120 clade C of strain ZA.1197MB, gp41 clade C of strain ZA.1197MB, C-terminal 6xHis 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 phycoerythrin (PE) mouse IgG1-IgG4 secondary antibodies.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, California, USA). Mann-Whitney U tests were used for the comparisons between any 2 groups. Variation across multiple groups was assessed using Kruskal-Wallis H test or two-way ANOVA. The correlation between two variables were done using Spearman's rank correlation. P values were considered significant if less than 0.05.

Acknowledgements

We would like to acknowledge all study participants and HPP Laboratory staff and thank Miss Fatima Laher (HPP, University of KwaZulu-Natal, South Africa) for assistance with HLA class

II tetramer validation. We would also like to acknowledge the following funding sources; HHMI International research scholar award (Grant #55008743), The US National Institute of Health (R37 AI67073), Dan and Marjorie Sullivan Research scholar award (Grant # 224910), the support of the National Research Foundation (NRF) through a Doctoral Innovation scholarship to O.B. (2014-2016) and the South African Research Chairs Initiative. Additional funding was from the Mark and Lisa Schwartz Foundation, the Bill and Melinda Gates Foundation, the International AIDS Vaccine Initiative (IAVI), grant number UKZNRSA1001 and the Victor Daitz Foundation. This work was also partially supported by Gilead Sciences Incorporated and the Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE), a DELTAS Africa Initiative (grant # DEL-15-006). The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)'s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa's Development Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome Trust (grant # 107752/Z/15/Z) and the UK government. The views expressed in this publication are those of the author(s) and not necessarily those of AAS, NEPAD Agency, Wellcome Trust or the UK government.

Conflict of interest

The authors declare no conflict of interest.

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629

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633 *Data are represented as median (IQR).

634 **Table 2**: Study participants for tetramer staining assay

635 *Data are represented as median (IQR).

636

637 **Figure legends**

638

639 **Figure 1:** Memory distribution of $CXCR5^+$ cells within circulating $CD4^+$ T cell compartment in
640 healthy donors. (A) Representative flow cytometry plot showing the gating strategy for $CD4^+$ T
641 cell memory populations. (B) Summary dot plots showing proportions of $CD4^+$ T cells that are
642 naïve, central (CM), effector (EM) and terminally differentiated (TEMRA) memory cells. (C)
643 Pie chart showing median percentages of $CXCR5^+$ and $CXCR5^-$ $CD4^+$ T cells. (D)
644 Representative flow cytometry plots for $CXCR5^+$ and $CXCR5^-$ gating within bulk $CD4^+$ T cells
645 and summary plots depicting the proportions of $CXCR5^+$ (blue) and $CXCR5^-$ (red) $CD4^+$ T cells
646 within the CM, EM, naïve and TEMRA memory subsets. Statistical analysis was done using
647 Kruskal-Wallis H test (B) and Mann-U Whitney tests (D).

648

649 **Figure 2:** Heterogeneity within circulating Tfh compartment during acute HIV-1 infection. (A)
650 Representative flow cytometry plots showing the gating strategy for bulk cTfh within CD45RA-
651 $CD4^+$ T cells and summary proportions of cTfh cells in HIV negative and acute HIV groups. (B)
652 Summary plot comparing the frequencies of naïve, CM, EM and TEMRA cTfh cells in HIV
653 negative and acute HIV donors. (C) Gating strategy for Tfh1, Tfh1-17, Tfh2 and Tfh17 subsets.
654 The proportions of Tfh1, Tfh2, Tfh1-17 and Tfh17 subsets are compared between HIV negative
655 and acute HIV groups. P values are from Mann-U Whitney tests.

656

657 **Figure 3:** Tfh1 correlates negatively with set point viral load. Set point viral load was plotted
658 against the frequency percentages of (A) Tfh1, (B) Tfh2, (C) Tfh1-17, (D) Tfh17 and (E) bulk
659 cTfh cells determined by flow cytometry. Spearman rho (r) values and p values are reported.
660
661 **Figure 4:** Early Tfh1 responses are predictive of p24 IgG responses at 1 year of infection. (A)
662 p24 IgG titers, (B) gp41 IgG titers, (C) p17 IgG titers, (D) gp120 IgG titers and (E) p24 IgG1
663 titers, at 1 year time-point were determined using a customized multivariate Luminex assay and
664 the values were inversely correlated to SPVL. (F) p24 IgG, (G) p17 IgG, (H) gp41 IgG, (I) p24
665 IgG1 and (J) gp120 IgG titers at 1 year time-point were correlated to the frequencies of Tfh1 at
666 6.9 (IQR, 5.0-8.0) weeks of infection. Mean Fluorescence Intensity (MFI) and viral load (VL).
667 Spearman rho (r) values and p values are reported.
668
669 **Figure 5:** HIV specific cTfh measurements using ICS assay. (A) Representative flow cytometry
670 plots for cytokine secreting cTfh cells. PBMCs were unstimulated or stimulated with SEB or
671 HIV OLP pools for Gag, Nef and Env for 16h in the presence of GolgiStop and GolgiPlug
672 transport inhibitors (BD Biosciences), and the intracellular expression of IL-21, IL-4, TNF-α
673 and IFN-γ respectively was measured. (B) Summary frequency plots for Gag, Nef and Env-
674 specific cTfh cells. (C) Summary plots for SEB stimulated cells. (D) Total HIV-specific cTfh
675 cells. IL-21$^+$, IL-4$^+$, TNF-α$^+$ and IFN-γ^+ cTfh cells were summed up for Gag, Nef and Env. (E)
676 Comparison of the cytokine secretion profiles of cTfh (CXCR5$^+$) and non-cTfh (CXCR5$^-$) cells.
677 Frequencies for Gag (i), Nef (ii) and Env-specific (iii) cells were plotted separately or totaled
678 (iv). P values are from Mann-U Whitney test.
679

680 **Figure 6:** HIV specific cTfh detection by HLA class II tetramers. (A) Representative flow
681 cytometry plots showing gating strategy for tetramer double positive (Tet++) cells within $CD4^+$
682 T cells and $CD8^+$ T cells. (B) HIV specific cTfh cells (in patient 1) are detected at 12, 14, 16 and
683 20 weeks (patient 1) or at (C) 6 and 138 weeks post-infection (patient 2) using HLA class II
684 tetramers. Overlay of Tet++ cells (red) onto $CXCR5^+CXCR3^+CD4^+$ cTfh. $CXCR3^+CXCR5^+$
685 gate (black) and $CXCR3^-CXCR5^+$ gate (green). (D) Summary plot comparing the frequencies
686 of $CXCR3^+$ (Tfh1 & Tfh1-17) and $CXCR3^-$ (Tfh2 & Tfh17) tetramer specific cTfh cells. P
687 value is from Mann-U Whitney test.
688

Table 1: Characteristics of study participants

	HIVneg	**Acute HIV**
n	12	14
Male	0	6
Female	10	8
CD4 Count (cells/µl)*	N/A	463 (422-561)
Viral load (copies/ml)*	N/A	121 000 (8 984-352 000)
Time post-infection (weeks)*	N/A	6.9 (5.0-8.0)

*Data are represented as median (IQR).

Table 2: Study participants for tetramer staining assay

	Acute HIV	**Chronic HIV**
n	2	5
Male	0	0
Female	2	5
CD4 Count (cells/µl)*	365 (351-433)	720 (537-1 022)
Viral load (copies/ml)*	18 760 (9 642-377 905)	2950 (455-30 975)

*Data are represented as median (IQR).

Figure 1.

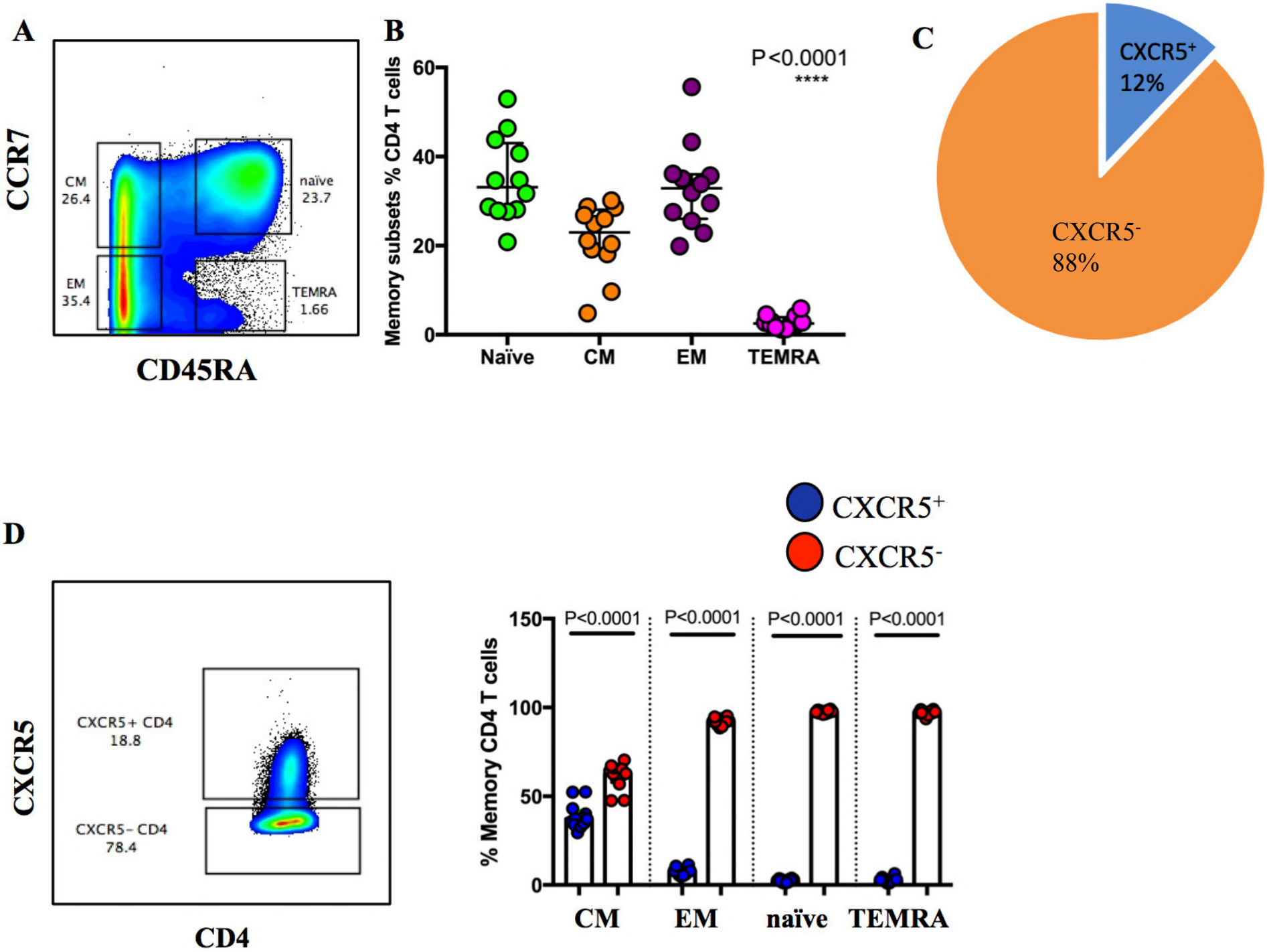

A
CCR7
CD45RA
CM
26.4
naïve
23.7
EM
35.4
TEMRA
1.66
B
Memory subsets % CD4 T cells
60
40
20
0
P<0.0001
****
Naïve
CM
EM
TEMRA
C
CXCR5+
12%
CXCR5-
88%
D
CXCR5
CD4
CXCR5+ CD4
18.8
CXCR5- CD4
78.4
CXCR5+
CXCR5-
% Memory CD4 T cells
150
100
50
0
P<0.0001
P<0.0001
P<0.0001
P<0.0001
CM
EM
naïve
TEMRA

Figure 2.

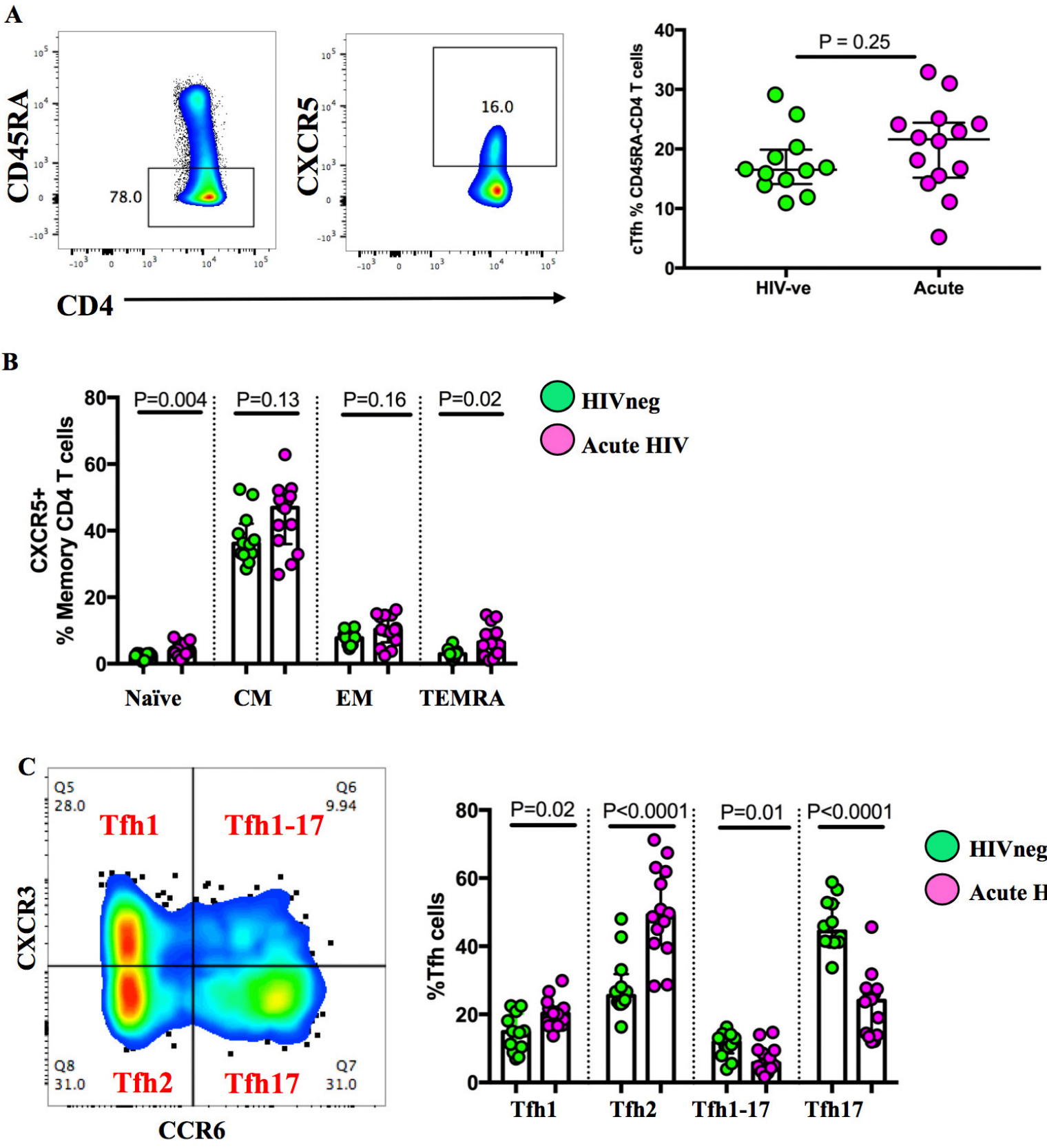


Figure 3.

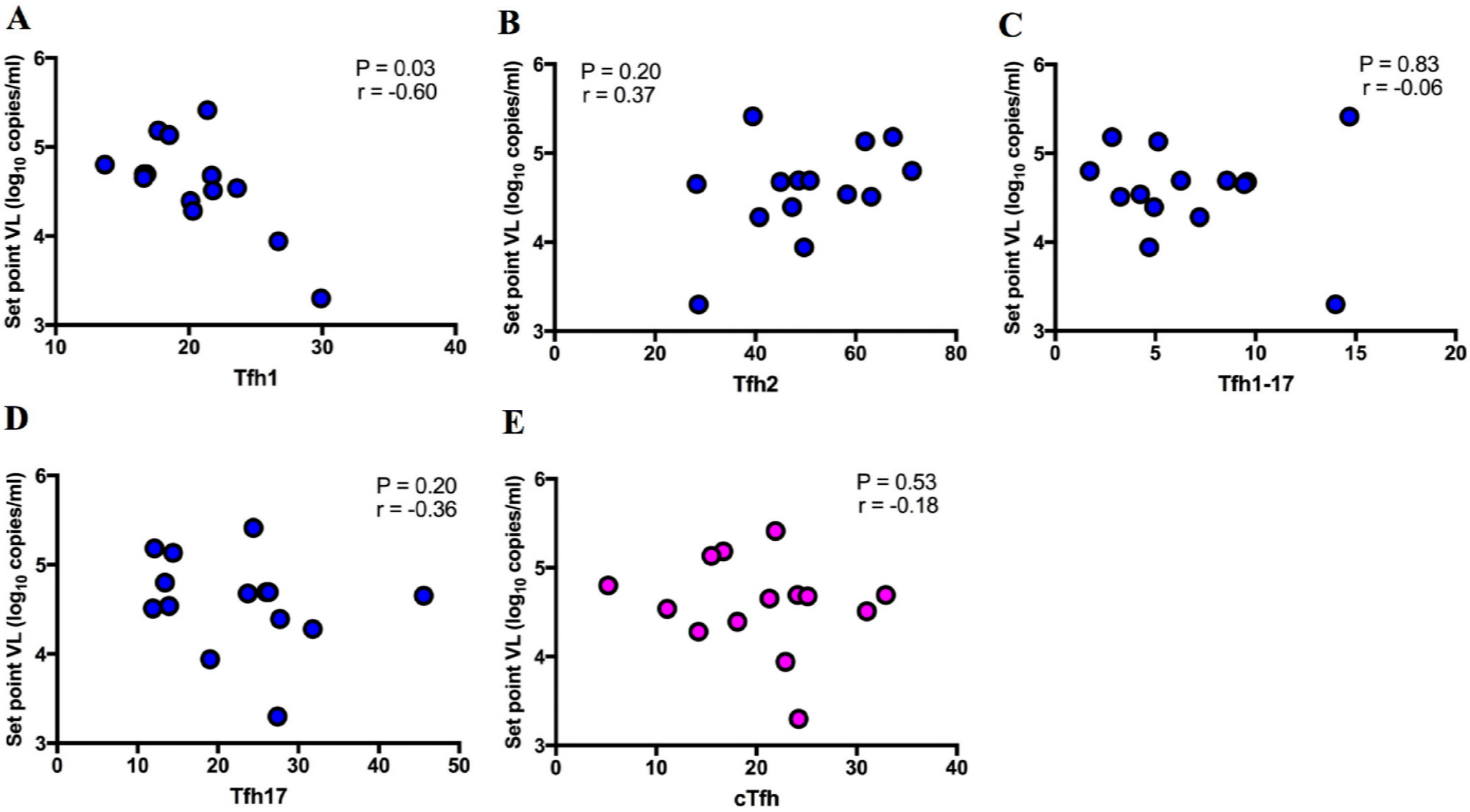
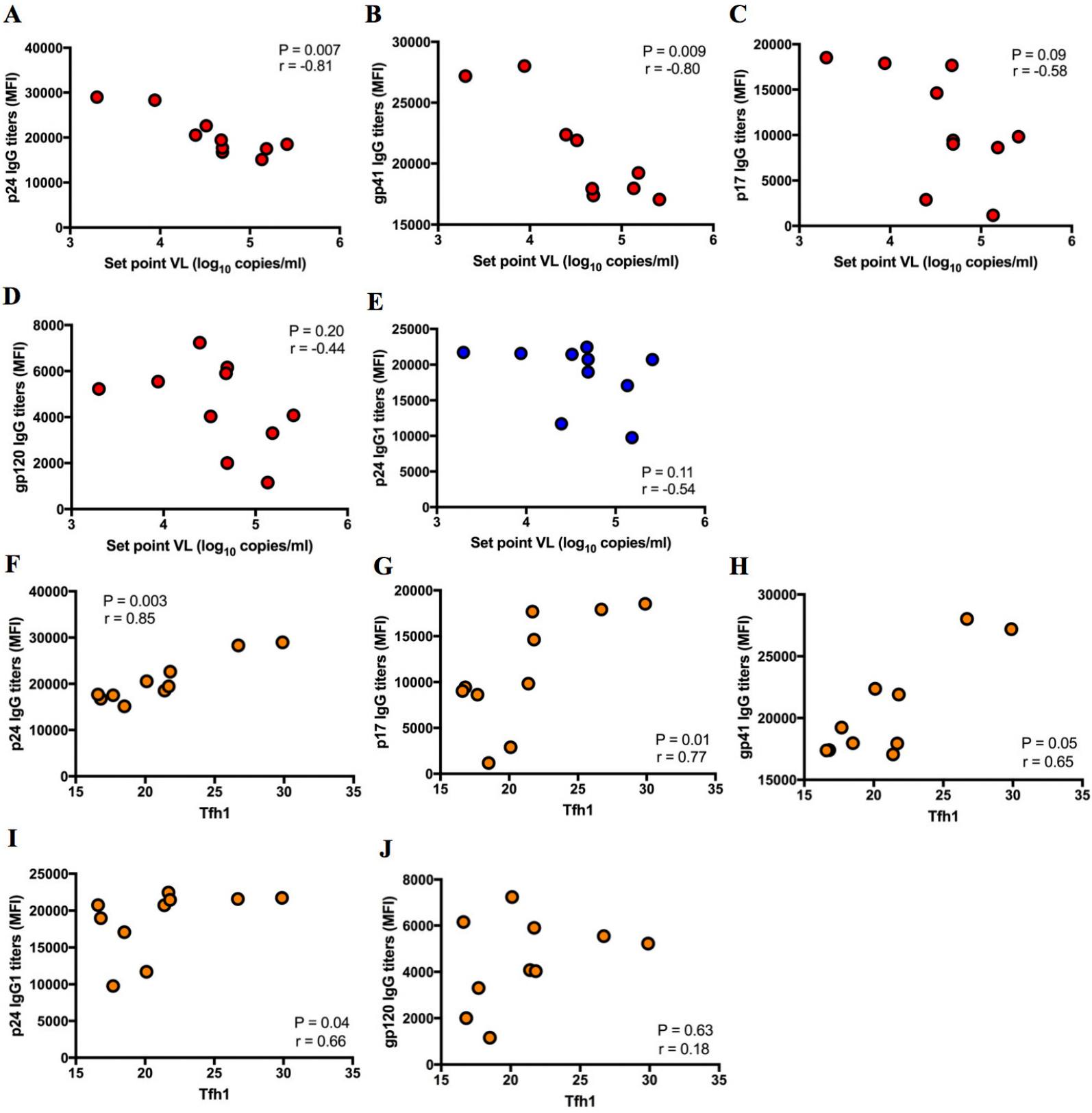

A
P = 0.03
r = -0.60
Set point VL (log10 copies/ml)
Tfh1
B
P = 0.20
r = 0.37
Set point VL (log10 copies/ml)
Tfh2
C
P = 0.83
r = -0.06
Set point VL (log10 copies/ml)
Tfh1-17
D
P = 0.20
r = -0.36
Set point VL (log10 copies/ml)
Tfh17
E
P = 0.53
r = -0.18
Set point VL (log10 copies/ml)
cTfh

Figure 4.



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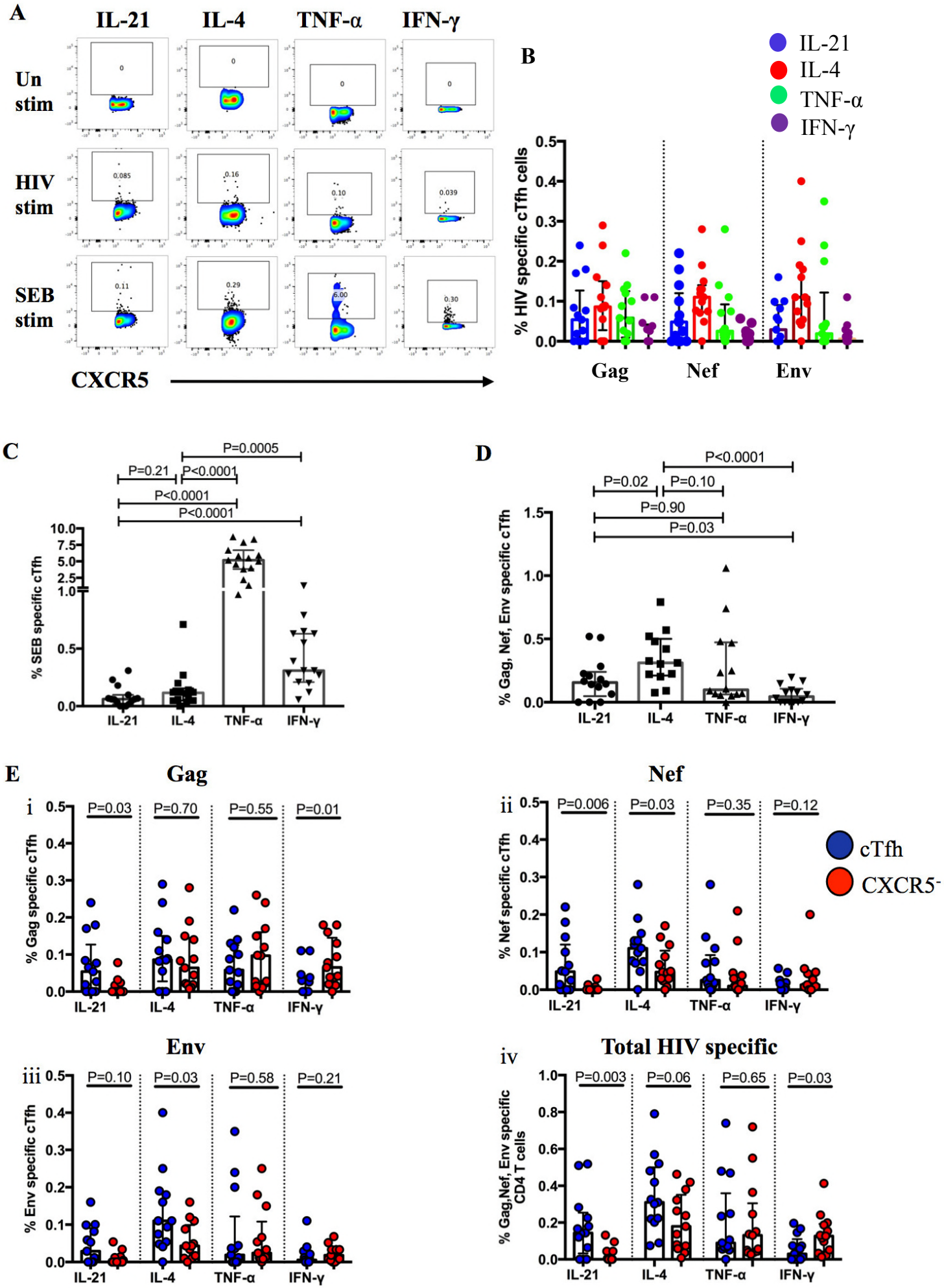
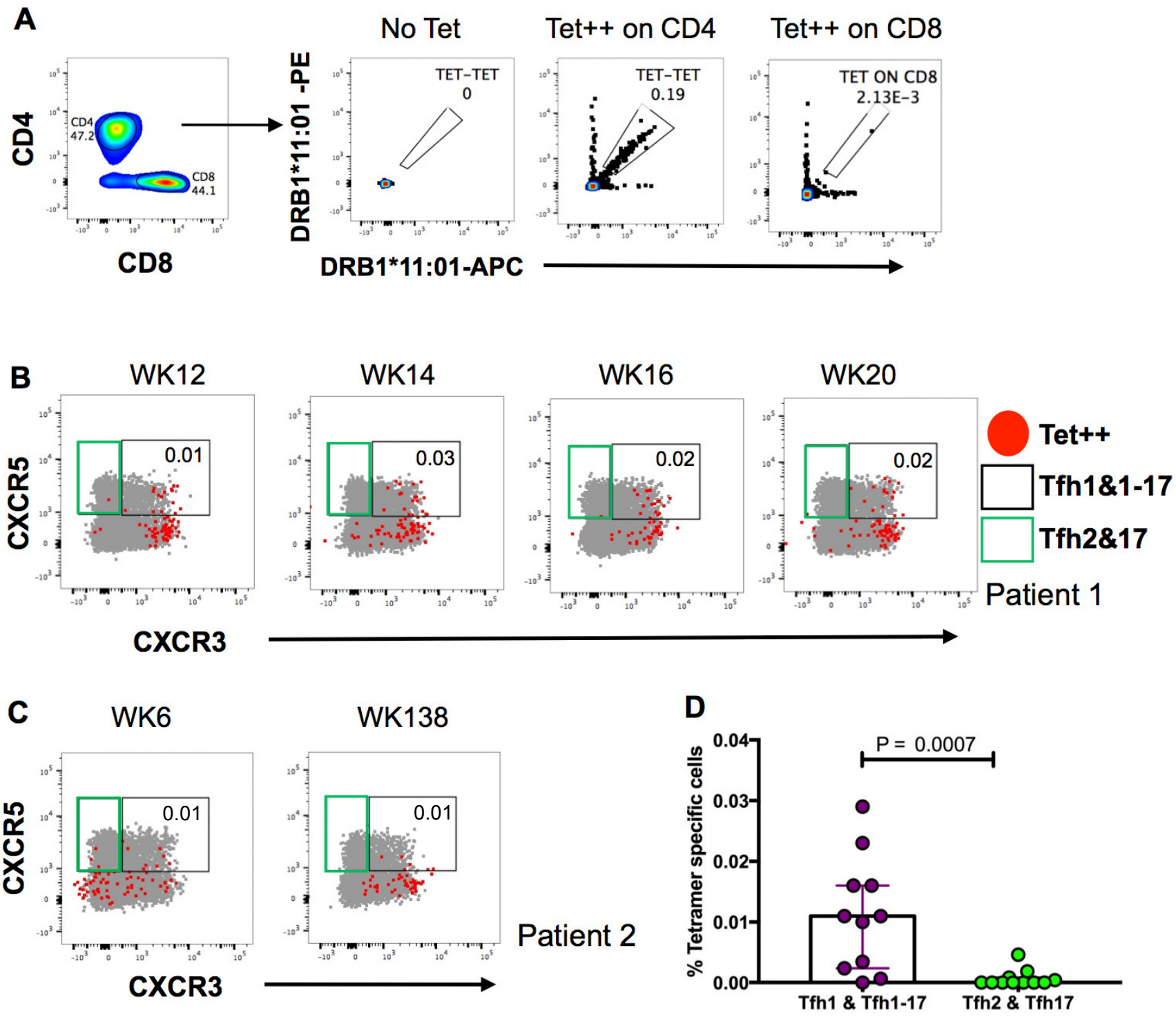


Figure 6.


A
No Tet
Tet++ on CD4
Tet++ on CD8
CD4
47.2
CD8
44.1
CD8
DRB1*11:01 -PE
TET-TET
0
TET-TET
0.19
TET ON CD8
2.13E-3
DRB1*11:01-APC
B
WK12
WK14
WK16
WK20
CXCR5
0.01
0.03
0.02
0.02
Tet++
Tfh1&1-17
Tfh2&17
Patient 1
CXCR3
C
WK6
WK138
CXCR5
0.01
0.01
Patient 2
CXCR3
D
% Tetramer specific cells
0.04
0.03
0.02
0.01
0.00
P = 0.0007
Tfh1 & Tfh1-17
Tfh2 & Tfh17