Loss of Circulating CD4 T Cells with B Cell Helper Function during Chronic HIV Infection

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Abstract

The interaction between follicular T helper cells (Tfh) and B cells in the lymph nodes and spleen has a major impact on the development of antigen-specific B cell responses during infection or vaccination. Recent studies described a functional equivalent of these cells among circulating CD4 T cells, referred to as peripheral Tfh cells. Here, we characterize the phenotype and in vitro B cell helper activity of peripheral Tfh populations, as well as the effect of HIV infection on these populations. In coculture experiments we confirmed CXCR5+ cells from HIV-uninfected donors provide help to B cells and more specifically, we identified a CCR7highCXCR5highCCR6highPD-1high CD4 T cell population that secretes IL-21 and enhances isotype-switched immunoglobulin production. This population is significantly decreased in treatment-naive, HIV-infected individuals and can be recovered after anti-retroviral therapy. We found impaired immunoglobulin production in co-cultures from HIV-infected individuals and found no correlation between the frequency of peripheral Tfh cells and memory B cells, or with neutralization activity in untreated HIV infection in our cohort. Furthermore, we found that within the peripheral Tfh population, the expression level of Tfh-associated genes more closely resembles a memory, non-Tfh population, as opposed to a Tfh population. Overall, our data identify a heterogeneous population of circulating CD4 T cells that provides in vitro help to B cells, and challenges the origin of these cells as memory Tfh cells.

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Introduction

Follicular helper CD4 T cells (Tfh) are crucial for the development of antigen-specific B cells within germinal centers (GC). Tfh cells interact through co-stimulatory receptors and provide essential soluble factors (i.e. IL-4, IL-21) to promote the survival, isotype switching and selection of high affinity memory B cells [1]. Phenotypic and gene signature analysis has revealed a highly conserved molecular profile of Tfh cells in humans, non-human primates (NHP) and mice, which is characterized by increased expression of Bcl-6, CXCR5, PD-1, ICOS and decreased expression of CCR7 [2-4]. Human Tfh cells exhibit a polarized cytokine profile characterized by compromised production of TH1 cytokines and increased secretion of IL-4, IL-10 and IL-21 [5]. Although IL-21 is characterized as a “hallmark” cytokine of Tfh cells, other THelper subsets produce this cytokine [6]. The origin and differentiation of Tfh is unclear, as previous studies found Tfh cells can derive from TH1 or TH2 cells, or independently of other CD4 lineages [7-9]. However, it is well established that the transcription factor Bcl-6 regulates several molecules involved in Tfh development (i.e. PD-1, IL-21R, CXCR5) [10,11]. Similarly, the fate of Tfh, particularly those in the germinal center (GC-Tfh), following the effector phase of the immune response is unclear. We have recently shown that NHP GC-Tfh display compromised in vivo cell cycling and are prone to in vitro cell death [4]. Other studies have shown that Tfh can form a memory pool found in anatomical sites outside the lymph nodes [12]. Hence, Tfh cells may adopt a “central memory” phenotype or undergo cell death after the effector phase [13]. In
Humans, a circulating CD4 T cell population characterized by high CXCR5 expression can provide in vitro help for B cell isotype switching and shares functional characteristics with TFH cells [14]. It was proposed that these circulating cells, termed “peripheral TFH” (pTFH) could represent the memory counterparts of TFH outside the lymphoid organs. Further investigation is needed to establish a direct relationship between TFH cells and pTFH cells.

It is becoming increasingly important to understand the interplay between CD4 T cells and B cells during HIV infection, specifically with relation to the generation of broadly neutralizing antibodies. Chronic HIV/SIV infection results in profound changes in CD4 T cell dynamics in lymph nodes characterized by TFH accumulation and increased ability of non-TFH to egress the lymph node [4,15]. How this impacts upon the dynamics of pTFH is unknown. Elucidating the biology and dynamics of pTFH, and their ability to provide B cell help may be important for our investigation [16]. Notably, our sorted naive B cell population did not express isotype-switched immunoglobulin (Figure S1A) and culture conditions that lacked SIV did not induce immunoglobulin production (data not shown). Naive and CM CCR7low CD4 T cells failed to promote B cell differentiation and immunoglobulin production whereas CM CCR7highCXCR5highCD150+CD4 T cells induced limited production of IgM, IgG1 and IgG3 compared to the CCR7highCXCR5highCD150+CD4 T cell population (Figure 1B). The CCR7highCXCR5highCCR6highPD-1high population induced the greatest production of IgG1, IgG3 and IgA compared to the CCR7highCXCR5highCD150+CD4 T cell population. Prior studies defined pTFH cells based on surface expression of CXCR5, CCR6 and the lack of CCR3 expression [14]. We found that the greatest help for immunoglobulin production was from CCR3lowCCR6lowCD4 T cell populations and, within those, from the PD-1high cells. We did not eliminate a small population of CCR3+ cells in order to avoid removing a larger population of CCR3highCCR6highCD4 T cells that induce B cell differentiation (Figure S1B).

The cytokine profile of pTFH populations shared characteristics with other T_helfer subsets, including T_H1, T_H2, and T_reg cells. Supernatant from the CCR3lowCCR6lowPD-1high coculture contained the greatest quantities of TNF-α, IL-2, and IL-17 compared to the CCR3highCCR6highPD-1high coculture (Figure 1C). Notably, the CCR3highCCR6highPD-1high population, which promoted the greatest production of IgG1, IgG3 and IgA, showed the greatest IL-21 production, although at low levels.

Overall, CCR3high CD4 T cell populations induced B cell immunoglobulin production, although the CCR3lowCCR6lowPD-1high population did so most efficiently. However, this population is not characteristic of a TFH population found in secondary lymphoid organs, as coculture supernatants included a broad array of cytokines characteristic of TFH cells and multiple other T_helfer subsets.
Figure 1. Characterization of peripheral T<sub>fh</sub> cells. (A) Left: Representative flow cytometry plots from HIV-uninfected PBMC showing the gating scheme for isolating T cell subsets for the T cell/B cell coculture assay. Isolated populations include naïve cells (brown), CM CCR<sup>7</sup>low (pink), CM CCR<sup>7</sup>highCXCR<sup>5</sup>low (orange), CM CCR<sup>7</sup>highCXCR<sup>5</sup>highCCR<sup>6</sup>lowPD-1<sup>high</sup> (green), CM CCR<sup>7</sup>highCXCR<sup>5</sup>highCCR<sup>6</sup>highPD-1<sup>low</sup> (blue) and CCR<sup>7</sup>highCXCR<sup>5</sup>highCCR<sup>6</sup>highPD-1<sup>high</sup> (red). Before gating on CCR6 and PD-1, cells were first gated on CD150<sup>high</sup>. Right: Scatter plot indicating the frequency of each
population in HIV-uninfected subjects (n = 13). Cells were not gated on CD150 for phenotypic analysis. (B) Indicated CD4 T cell populations were cultured with autologous naïve B cells (CD19highCD27lowIgD−) in the presence of SEB for 12 days and Ig concentrations were measured from supernatants (n = 6). (C) Indicated CD4 T cell populations were cultured with autologous naïve B cells in the presence of SEB for 2 days and cytokine concentrations were measured from supernatants (n = 6). Horizontal lines indicate limit of detection. Significant differences were determined using the Friedman test with Dunn’s multiple comparison post-test. *p<0.05, **p<0.01.

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Progressive loss of pTFH cells in HIV infection

To determine the impact of HIV on pTFH populations, we compared pTFH cells from HIV-uninfected subjects and treatment-naive HIV-infected subjects (Table S1) as a frequency of total CD4 cells. Irrespective of how pTFH cells were defined, there was a significant decrease in the pTFH population from HIV-infected subjects compared to HIV-uninfected subjects (Figure 2A). Subjects with CD4 counts greater than 200 had significantly lower pTFH populations, while subjects with CD4 counts less than 200 had the lowest frequency of all phenotypically defined pTFH populations. However, when we defined the CCR6highPD-1high population as a subset of the CCRX5high population, the frequency of the CCR6highPD-1high population increased in subjects with CD4 counts less than 200 (Figure S2A). The increase in PD-1high cells was likely due to immune activation in HIV infection, as we observed increases in the frequency of both PD-1high and ICOShigh cells within the CCRX5high population, with the greatest increases seen in samples with CD4 counts less than 200 (Figure S2A). We also observed a positive trend between CCRX5highPD-1high cells and serum concentrations of soluble CD14. (Figure S2A). For 10 HIV-infected individuals on whom we had longitudinal samples, we observed a loss of pTFH populations as a frequency of total CD4 T cells over 36 to 48 months (Figure 2B). However, the frequency of PD-1high, ICOShigh and CCR6highPD-1high cells within the CCRX5high population remained stable (Figure S2B).

Next, we investigated the impact of ART on the frequency of pTFH within total CD4 T cells. Longitudinal analysis on samples from before and after 24 and 48 weeks of ART revealed a recovery of pTFH populations (Figure 2C). However, the frequency of PD-1high, ICOShigh and CCR6highPD-1high cells remained stable within the CCRX5high population (Figure S2C). Overall, HIV infection causes a loss of pTFH cells and ART promotes the recovery of these populations.

Defective cytokine production of pTFH in HIV infection

To analyze HIV-specific cells, PBMC were stimulated with Gag peptide pools and analyzed for cytokine expression. Very few IL-2-positive and IL-17-positive cells were detected within the CM compartment (data not shown). Gag-specific IFN-γ and IL-21-producing cells were detected, however, compared to SEB-stimulation fewer HIV-specific cells expressed CCR7 (4.4% vs 10.7% of IFN-γ positive cells; 3.5% vs 11.9% of IL-21-positive cells for Gag and SEB stimulation, respectively). A majority of HIV-specific cells were not CCR7highCXCR5highCCR6high (Figure 4C; 0.4% of IFN-γ positive cells and 0.9% of IL-21-positive cells were CCR7highCXCR5highCCR6high).
Figure 2. Progressive loss of pTFH cells in HIV infection. (A) Pooled data showing the frequency (%) of CXCR5<sup>high</sup>, CXCR5<sup>high</sup>CCR6<sup>high</sup> and CXCR5<sup>high</sup>CCR6<sup>high</sup>PD-1<sup>high</sup> populations in total CD4 cells from PBMC from HIV uninfected (open circles; *n = 13), HIV-infected (treatment-naive), CD4 count >200 (light gray circles; *n = 44), and HIV-infected (treatment-naive), CD4 count <200 (black circles; *n = 22). Significant differences between HIV-uninfected and HIV-infected subjects were determined using the Mann-Whitney U test. ***p < 0.001; **p < 0.01; *p < 0.05. (B) Longitudinal analysis showing the frequency (%) of CXCR5<sup>high</sup>, CXCR5<sup>high</sup>CCR6<sup>high</sup> and CXCR5<sup>high</sup>CCR6<sup>high</sup>PD-1<sup>high</sup> populations in total CD4 cells or indicated populations in CXCR5-expressing cells (bottom row) from HIV-infected (treatment naive) subjects (*n = 10) over 36–48 months. No significant correlations were found. (C) Pooled data showing the frequency (%) of CXCR5<sup>high</sup>, CXCR5<sup>high</sup>CCR6<sup>high</sup> and CXCR5<sup>high</sup>CCR6<sup>high</sup>PD-1<sup>high</sup> populations in total CD4 cells from PBMC from HIV-uninfected subjects (open circles; *n = 13) and HIV-infected subjects before (*n = 14, week 0; black circles) and after ART (week 24, dark gray circles) and after 24 weeks of ART (gray circles).
Loss of Peripheral T<sub>FH</sub> Cells during HIV Infection

Overall, we observed IL-21 production from the CCR<sup>7</sup><sub>high</sub>CCR<sup>5</sup><sub>high</sub>CCR<sup>6</sup><sub>high</sub> pT<sub>FH</sub> population, although we detected the most IL-21 in non-pT<sub>FH</sub> cells, which were CCR<sup>7</sup><sub>low</sub> and CCR<sup>5</sup><sub>low</sub>. In addition to IL-21, the CCR<sup>7</sup><sub>high</sub> CCR<sup>5</sup><sub>high</sub>CCR<sup>6</sup><sub>high</sub> pT<sub>FH</sub> population produced IL-2 and IL-17, cytokines characteristic of T<sub>FH</sub> and T<sub>H</sub>17 cells, respectively. However, from HIV-infected individuals we observed a loss of CCR<sup>7</sup><sub>high</sub>CCR<sup>5</sup><sub>high</sub>CCR<sup>6</sup><sub>high</sub> cells making IL-2, IL-17 and IL-21.

No relationship between pT<sub>FH</sub> cells and neutralization activity

Previous studies have described a relationship between the frequency of peripheral CXCR<sup>5</sup><sub>high</sub> cells and memory B cells and antibody titers with vaccination [16]. Therefore, we analyzed the relationship between the frequency of pT<sub>FH</sub> cells and IgG-positive memory B cells in PBMC from HIV-infected individuals. We found no significant correlation between the frequency of pT<sub>FH</sub> cells and IgG-positive B cells (Figure 5A). Similarly, we failed to detect a relationship between the frequency of pT<sub>FH</sub> and HIV-1 Env-specific antibody titers or total plasma IgG levels (data not shown).

It has also been reported that PD-1<sup>high</sup> CD4<sup>+</sup> T cells in blood are associated with cross-clade neutralizing antibody responses during HIV infection [19] and these PD-1<sup>high</sup> CD4<sup>+</sup> T cells may represent a population of pT<sub>FH</sub> cells. Thus, the relationship between pT<sub>FH</sub> cells and neutralization activity was analyzed using HIV-infected samples classified as good neutralizers (median ID<sub>50</sub>&gt;100) or poor neutralizers (median ID<sub>50</sub>&lt;100) [20]. Irrespective of how pT<sub>FH</sub> cells were defined, we failed to find any relationship between neutralization activity and pT<sub>FH</sub> cells (Figure 5B).

Relationship between pT<sub>FH</sub> cells and T<sub>FH</sub> cells in human tonsil

While pT<sub>FH</sub> cells induce B cell differentiation and immunoglobulin secretion in vitro, the relationship between pT<sub>FH</sub> and T<sub>FH</sub> cells in secondary lymphoid organs remains unclear. Our in vitro coculture studies indicated the greatest isotype-switched immunoglobulin production was elicited from B cells cocultivated with CXCR<sup>5</sup><sub>high</sub>CCR<sup>5</sup><sub>high</sub> pT<sub>FH</sub> cells (Figure 1B). Therefore, we investigated the expression of CCR6 on T<sub>FH</sub> (CXCR<sup>5</sup><sub>high</sub>PD-1<sup>high</sup>) and non-T<sub>FH</sub> (CXCR<sup>5</sup><sub>low</sub>PD-1<sup>low</sup>) tonsil cells to determine if the CXCR<sup>5</sup><sub>high</sub>CCR<sup>5</sup><sub>high</sub> pT<sub>FH</sub> population is related to T<sub>FH</sub> cells within secondary lymphoid organs (Figure 6A). The lowest frequency of CXCR<sup>5</sup><sub>high</sub> cells was found within the CXCR<sup>5</sup><sub>high</sub>PD-1<sup>high</sup> compartment (1.5% of CXCR<sup>5</sup><sub>high</sub>PD-1<sup>high</sup> cells) and the greatest frequency of CXCR<sup>5</sup><sub>high</sub> cells within the non-T<sub>FH</sub> compartment (9% of CXCR<sup>5</sup><sub>low</sub>PD-1<sup>low</sup> cells; Figure 6B).

Similarly, RNA sequence data from the CXCR<sup>5</sup><sub>high</sub>CCR<sup>5</sup><sub>low</sub>PD-1<sup>low</sup> pT<sub>FH</sub> population more closely resembles a memory, non-T<sub>FH</sub> CD4<sup>+</sup> T cell population from the tonsil (CM CD57<sup>low</sup>PD-1<sup>dim</sup>CCR<sup>7</sup><sub>high</sub>CCR<sup>5</sup><sub>low</sub>CXCR<sup>4</sup><sub>low</sub>) as compared to the non-germinal center T<sub>FH</sub> population (CM CD57<sup>low</sup>PD-1<sup>dim</sup>CCR<sup>7</sup><sub>low</sub>CCR<sup>5</sup><sub>low</sub>CXCR<sup>4</sup><sub>low</sub>) or the GC-T<sub>FH</sub> upon re-exposure to antigen, or both. Our studies begin to address these issues by further defining pT<sub>FH</sub> cells, comparing pT<sub>FH</sub> cells to tonsillar T<sub>FH</sub> cells, and analyzing the effect of HIV on these cells.

Discussion

The development and nature of human T<sub>FH</sub> memory cells following an effector immune response are not known. The ability to define a population of memory T<sub>FH</sub> cells in PBMC (pT<sub>FH</sub>) would help inform our understanding of CD4<sup>+</sup> T cell dynamics within lymphoid tissue during vaccination or infection. Studies of chronic infection may be helpful in this regard [21]. Whether the accumulation of T<sub>FH</sub> cells during chronic infection [4,15] impacts the T<sub>FH</sub> memory population is of particular interest, especially if memory T<sub>FH</sub> cells migrate between lymphoid organs and peripheral tissues. Recent studies [14,16] have suggested that circulating CXCR<sup>5</sup><sub>high</sub> CD4<sup>+</sup> T cells may represent the peripheral counterparts of T<sub>FH</sub> cells. However, the relationship between pT<sub>FH</sub> and T<sub>FH</sub> cells within secondary lymphoid organs remains unclear. Therefore, it is of great relevance to determine if pT<sub>FH</sub> cells originate from GC-T<sub>FH</sub> cells and represent a memory T<sub>FH</sub> population, reflect a precursor population that differentiates into GC-T<sub>FH</sub> upon re-exposure to antigen, or both. Our studies begin to address these issues by further defining pT<sub>FH</sub> cells, comparing pT<sub>FH</sub> cells to tonsillar T<sub>FH</sub> cells, and analyzing the effect of HIV on these cells.

In concordance with previous studies, we showed that circulating CXCR<sup>5</sup><sub>high</sub> CD4<sup>+</sup> T cells support B cell differentiation in vitro [14,17]. A majority of the CXCR<sup>5</sup><sub>high</sub> cells expressed CD150, and while CD150 was used for gating in the co-culture assays, we found it did not impact the loss of pT<sub>FH</sub> cells or effect our results with respect to loss of pT<sub>FH</sub> cells, recovery with ART or lack of association with B cell or antibody responses (data not shown). However, within the CXCR<sup>5</sup><sub>high</sub> population the expression of CCR6 and PD-1 did further define pT<sub>FH</sub> populations with differential abilities for naïve B cell help and isotype switching. Thus, pT<sub>FH</sub> cell populations support both the activation and maturation of naïve B cells, and immunoglobulin isotype switching. Correspondingly, the individual pT<sub>FH</sub> populations produced cytokines associated with B cell maturation and survival, such as IL-21 [22], IL-2 [23] and IL-17 [24], in contrast to T<sub>FH</sub> cells within secondary lymphoid tissue, which display a limited cytokine profile that includes IL-4, IL-10 and IL-21, but compromised production of IL-2 and IL-17 [4]. Whether these pT<sub>FH</sub> populations represent different stages of T<sub>FH</sub> memory development or originate from separate CD4<sup>+</sup> T cell populations within lymphoid tissue [25] is still unclear.
Figure 3. Impaired B cell help by pTFH cells in HIV infection. (A) CCR7<sup>hi</sup>CXCR5<sup>lo</sup> and CCR7<sup>hi</sup>CXCR5<sup>hi</sup>CCR6<sup>hi</sup> CM CD4 T cells isolated from PBMCs were cultured with autologous naive B cells (CD19<sup>hi</sup>CD27<sup>lo</sup>IgD<sup>−</sup>) in the presence of SEB for 12 days and Ig concentrations were measured from supernatants (HIV-uninfected, n = 8; HIV-infected (non-viremic), n = 5–7, HIV-infected (viremic), n = 1–2). Significant differences were determined using the Wilcoxon paired t-test or the Mann-Whitney test. *p < 0.05; **p < 0.01. (B) Top: HIV-uninfected PBMCs were incubated with indicated concentrations of CXCL-13 for 1 hour at 37°C (red) or 4°C (black). Bottom: Healthy PBMCs were incubated with 1 µg/mL CXCL13 for 10, 30,
In order to better understand the relationship between T<sub>FH</sub> and pT<sub>FH</sub> cells, we compared gene expression levels between pT<sub>FH</sub> and tonsillar CD4 T cell populations and focused on genes important for T<sub>FH</sub> differentiation, migration, and function. We found that the pT<sub>FH</sub> population with the greatest B cell helper function most closely resembled a CM, non-T<sub>FH</sub> CD4 T cell subset within the tonsil. While our studies do not directly address the relationship between GC-T<sub>FH</sub> in lymph nodes and circulating CD4 T cells from the same patients, our data challenge whether pT<sub>FH</sub> are memory T<sub>FH</sub> cells. A recent study reported that germinal center T<sub>FH</sub> cells in mice migrate throughout the follicle, but generally do not enter the follicle to enter the blood [26].

While it is conceivable that pT<sub>FH</sub> cells represent a minor population of T<sub>FH</sub> cells that exit the follicle, it is also possible that pT<sub>FH</sub> cells are reflective of a precursor T<sub>FH</sub> population that exits the lymphoid organ and enters the circulation before entering the follicle. However, while we find the CXCR<sup>5<sub>high</sub></sup>CCR<sup>6<sub>high</sub></sup>PD-1<sup>high</sup> pT<sub>FH</sub> population does not resemble a memory T<sub>FH</sub> population, Loci and colleagues found a CXCR<sup>5</sup>+CXCR3-PD-1+ pT<sub>FH</sub> subset that functionally and transcriptionally resembles a memory T<sub>FH</sub> population [27]. A recent study in mice reported that memory T<sub>FH</sub> cells have reduced mRNA expression of T<sub>FH</sub> markers such as Bcl6, IL-21, ICOS and PD-1 compared to the effector T<sub>FH</sub> population [28], indicating the expression of these molecules may change depending on the phase of infection.

Therefore, further investigation of pT<sub>FH</sub> subsets and their relationship to memory and effector populations at multiple stages of infection is needed.

pT<sub>FH</sub> and naive B cell co-cultures from HIV-infected subjects produced fewer immunoglobulins compared to co-cultures from HIV-uninfected subjects. The observed defect in immunoglobulin production is likely due to impaired pT<sub>FH</sub> help to B cells instead of B cell dysfunction, as co-cultures included naive B cells rather than memory B cells that exhibit abnormalities in HIV infection [29]. Furthermore, while co-culture supernatants from HIV-infected subjects demonstrated a heterogeneous cytokine profile, similar to HIV-uninfected subjects, intracellular cytokine staining showed a lower CCR<sup>7<sub>high</sub></sup>/CXCR<sup>5<sub>high</sub></sup>/CCR<sup>6<sub>high</sub></sup> pT<sub>FH</sub> cells produced IL-2, IL-17 and IL-21 in chronic HIV infection compared to HIV-uninfected individuals. Furthermore, gene expression analysis of HIV-infected pT<sub>FH</sub> revealed fewer IL-21 and IL-4 transcripts, although the overall levels of cytokine transcripts were low.

Recent studies have shown T<sub>FH</sub> cells within secondary lymphoid organs accumulate in some donors or animals during chronic HIV/SIV infection and that T<sub>FH</sub> accumulation is associated with GC B cell expansion and increased serum immunoglobulin concentrations [4,22,30]. In contrast to T<sub>FH</sub> cells, our studies revealed pT<sub>FH</sub> cells consistently decrease in chronic HIV infection, with disease progression resulting in a greater reduction of these compartments within the total CD4 T cell population. However, it should be noted that we were unable to analyze T<sub>FH</sub> cells within secondary lymphoid organs from these subjects and therefore we are unable to directly compare the frequency of pT<sub>FH</sub> cells and T<sub>FH</sub> cells from the same individual. The differences between the increase in T<sub>FH</sub> cells and decrease in pT<sub>FH</sub> cells may be due to differences in disease state (i.e. early vs late infection) or represent a steady state of T<sub>FH</sub> cells trafficking between the lymphoid tissue and the blood. The decreased frequency of pT<sub>FH</sub> in the blood may indicate impaired ability of T<sub>FH</sub> to exit the lymph node in chronic HIV infection where the tissue architecture is not intact. Alternatively, the decreased frequency of pT<sub>FH</sub> in the blood may be a result of pT<sub>FH</sub> trafficking to secondary lymphoid organs. In agreement with previous studies [14,17], we found a majority of CXCR<sup>5<sub>high</sub></sup> cells express CCR7, and it has previously been suggested that pT<sub>FH</sub> cells migrate to secondary lymphoid organs upon infection due their expression of CCR7 and CD62L [14].

A confounding factor with regard to how we interpret the decrease in pT<sub>FH</sub> cells is that we also found a reduction in the surface expression of CXCR5 on CD4 T cells in chronic HIV infection, which may result from increased sera levels of CXCL-13 [31,32]. Furthermore, our co-culture data indicate that CXCR<sup>5<sub>low</sub></sup>CD4 T cells from viremic subjects can induce some B cell differentiation. These data support the possibility that in chronic HIV infection, a subset of functional pT<sub>FH</sub> cells may be phenotypically defined as CXCR<sup>5<sub>low</sub></sup>. Additionally, it should be noted that analysis of cellular subsets within the CXCR<sup>5<sub>high</sub></sup> population in chronic HIV infection revealed the frequency of CCR<sup>R<sub>6<sub>high</sub></sup></sup>/PD-1<sup>high</sup> cells increased. These results are consistent with a state of generalized immune activation, as we also observed increased surface expression of ICOS on CXCR<sup>5<sub>high</sub></sup> and CXCR<sup>5<sub>high</sub></sup>/PD-1<sup>high</sup> cells, and a positive association between the frequency of PD-1<sup>high</sup> cells within the CXCR<sup>5<sub>high</sub></sup> population in chronic HIV infection and serum concentrations of soluble CD14 [33]. Similarly, gene expression analysis indicated increased transcript levels of activation markers, such as ICOS and CD69 within the pT<sub>FH</sub> population during HIV infection. Overall, these data emphasize the difficulty in defining pT<sub>FH</sub> cells in chronic HIV infection and understanding the relationship between pT<sub>FH</sub> cells and T<sub>FH</sub> cells.

The uncertain definition of pT<sub>FH</sub> cells in HIV infection may provide an explanation as to why we were unable to identify correlations between pT<sub>FH</sub> populations and circulating IgG-positive memory B cells, or between pT<sub>FH</sub> cells and HIV-specific IgG (data not shown). Furthermore, we found no correlation between the frequency of pT<sub>FH</sub> and the neutralization activity of a well-characterized cohort of HIV-infected donors [20]. However, the absence of a correlation between pT<sub>FH</sub> cells and circulating HIV Env-specific IgG may also be explained by the lack of a time-dependent association (early vs. late infection) between T<sub>FH</sub> and pT<sub>FH</sub> cells, or indicate that the generation of IgG and broadly neutralizing antibodies is regulated by parameters other than pT<sub>FH</sub>, confounded by T cell independent antibody production commonly observed in HIV infection [34] or generalized immune activation. Thus, our data challenge the application of the pT<sub>FH</sub> population as a surrogate of GC T<sub>FH</sub>-B cell interactions in chronic HIV infection. While our studies did not find a correlation between pT<sub>FH</sub> cells and neutralizing antibodies, several recent studies, each with a different definition of pT<sub>FH</sub> cells, have reported an association with antibody responses during vaccination, infection or autoimmune disease [27,35–37]. Therefore, further studies are needed to establish the association between pT<sub>FH</sub> subsets and the generation of neutralizing antibodies, especially in HIV infection.

Overall, our data indicate that a range of circulating CD4 T cell populations can provide B cell help, possibly through differential secretion of soluble factors and/or cell-cell contact interactions.
Loss of Peripheral T\(\text{FH}\) Cells during HIV Infection

**Figure A**
- **CD154\text{+} Cytokine\text{+} (HIV uninfected)**
- **CD154\text{+} Cytokine\text{+} (HIV infected)**
- **Central Memory**

**Figure B**
- **Gated on:** CM, CD154\text{+}, Cytokine\text{+}
- **IFN\(\gamma\)**
- **IL-2**
- **IL-17**
- **IL-21**

**Figure C**
- **Gated on:** CM, CD154\text{+}, Cytokine\text{+}
- **IFN\(\gamma\)**
- **IL-21**

Central Memory CD154\text{+} Cytokine\text{+} (HIV uninfected)
Central Memory CD154\text{+} Cytokine\text{+} (HIV infected)
Central Memory
and that HIV infection results in loss of these cells over time, but with relative increases within the CXCR5high compartment which may be explained by immune activation. Furthermore, we did not find any association between pTFH and measures of B cell function such as HIV neutralization breadth/potency, HIV-specific IgG, or total IgG, suggesting application of this population as a surrogate of GC T FH-B cell interactions during HIV infection may be limited. A better understanding of the differentiation process and the developmental relationship between pTFH subsets and lymph node T FH cells is critical for the establishment of reliable peripheral blood CD4 T cell correlates for monitoring infection- or vaccine-associated B cell responses.

Materials and Methods

Ethics statement

Signed informed consent was obtained in accordance with the Declaration of Helsinki and approved by the appropriate Institutional Review Board. Tonsil cells were acquired from anonymized discarded pathologic specimens from Children's National Medical Center (CNMC) under the auspices of the Basic Science Core of the District of Columbia Developmental Center for AIDS Research. The CNMC Institutional Review Board determined that study of anonymized discarded tonsils did not constitute ‘human subjects research.’

Subjects

Fresh HIV-uninfected peripheral blood mononuclear cells (PBMC) were obtained from individuals participating in the NIH research apheresis program. Fresh HIV-infected blood was obtained from the Vaccine Research Center Clinic or Drexel University College of Medicine. Frozen HIV-infected PBMC were obtained from three study populations (Table S1). For untreated HIV infection, cells were obtained from volunteers who participated in a therapeutic vaccination trial (no efficacy was observed) conducted in the 1990’s prior to the advent of combination antiretroviral therapy (cART) [38]. The second study population consisted of donors from a cohort used to identify individuals with HIV broadly neutralizing antibodies [20]. To study the effect of cART, we obtained PBMC from HIV-infected donors participating in AIDS Clinical Trials Group study A5142 prior to initiation of cART and 24 and 48 weeks post-therapy [39,40]. PBMC and

Figure 4. Functional characteristics of pTFH cells and the impact of HIV. (A) Representative flow cytometry plots showing CM, CD154-positive, cytokine-positive cells after SEB stimulation. CD154-positive, cytokine-positive CD4 T cells, shown by contour plots (blue: HIV-uninfected; red: HIV-infected), are overlaid onto 2 dimensional density plots for CM CD4 T cells plotted against CCR7 and CD3, and CXCR5 and CCR6. (B) Bar graphs showing the frequency of SEB-stimulated CD154-positive, cytokine-positive cells that express CCR7, CXCR5 and CCR6 (n = 5; Red: HIV-infected; n = 24). (C) Left: Gag-specific CD4+ T cells (CD154-positive, cytokine-positive) shown as red contour plots are overlaid onto 2 dimensional density plots for CM cells CD4 T cells plotted against CCR7 and CD3, and CXCR5 and CCR6. Right: Bar graphs showing the frequency of Gag-specific CD154-positive, cytokine-positive cells that express CCR7, CXCR5 and CCR6 (n = 14). *p<0.05.

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Figure 5. Relationship between pTFH cells and neutralization activity. (A) Correlative analysis showing the frequency (%) of CXCR5high, CXCR5highCCR6high and CXCR5highCCR6highPD-1high populations in total CD4 cells from HIV-infected (treatment naive) subjects (n = 50) versus the frequency of IgG+ B cells in the total B population. Correlations were analyzed using the nonparametric Spearman test. (B) Pooled data showing the frequency (%) of CXCR5high, CXCR5highCCR6high and CXCR5highCCR6highPD-1high populations in total CD4 cells based on neutralization activity (median ID50<100 or >100). No significant differences were determined.

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Figure 6. Relationship between pTFH cells and T* cells in human tonsil. (A) Representative flow cytometry plots from HIV-uninfected, pediatric tonsils showing the gating scheme for determining the frequency of CCR6high cells in T* cells (CXCR5highPD-1low) and non-T* populations. (B) Bar graphs showing the frequency of CCR6high cells in T* and non-T* populations in human tonsils (n = 5). (C) Heatmap analysis of selected genes from RNA-seq data comparing pTFH cells (CXCR5highCCR6highPD-1high) from HIV-uninfected donors, pTFH cells from HIV-infected donors, non-T* CD4 memory tonsil cells (CM CD57lowPD-1lowCCR7highCCR5lowCXCR4low), non-germinal center T* tonsil cells (CM CD57lowPD-1highCCR7lowCXCR5high) and germinal center T* tonsil cells (CM PD-1highCD57high) from HIV-uninfected donors. (D) Top: Comparison of MAF expression on CD4 T cells from blood or tonsil. Bottom: Geometric mean (MFI) of MAF expression in the indicated populations of central memory CD4 T cells normalized to MAF MFI in naïve CD4 T cells.

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tonsil cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin [Invitrogen].

Antibodies

Directly conjugated antibodies were acquired from the following: (1) BD Biosciences: CD3-H7APC, CXCR5-Alexa488 (RF8B2), CCR7-Alexa700, IgG-APC, IFN-γ-Alexa700 and IL-21-Alexa647 (3A3-N2,1) (2) Beckman Coulter: CD45RO-EDC and CD27-PC5 (3) Biologend: CCR7-BV421, CCR6-PE (TG7/ CCR6), CCR6-Alexa647 (TG7/CCR6), CD20-BV570, CD150-PE, IL-2-BV605, IL-17A-Cy5.5PerCP and CD154-Cy5.5PE (4) Invitrogen: CD4-Cy5.5PE, CD27-QD655, CD27-QD605 and CD19-PacBlue (5) Southern Biotech: IgD-FITC and IgD-PE (6) eBioscience: mAb-Fluor660 (sym0F1), CXCR5-PerCP-elfluor710 (M5U3UBE), Biotinylated anti-PD-1 was from R&D and streptavidin-Cy7PE, (or QD655) was from Molecular Probes. The following antibodies were conjugated in our lab: CD19-QD705 and CD57-QD565. Quantum dots and Aqua amine viability dye were obtained from Invitrogen.

Polychromatic flow cytometry

**Phenotypic analysis:** 1–2×10^6 PBMC were incubated with Aqua-dye and surface stained with titrated amounts of anti-CD3, anti-CD4, anti-CD27, anti-CD45RO, anti-CCR7, anti-CXCR5, anti-CD150, anti-CCR6, anti-PD-1 and anti-CD19. Post-wash, cells were incubated with fluorescent-conjugated streptavidin, washed and fixed with 1% paraformaldehyde.

**Intracellular cytokine staining:** 3×10^6 PBMC were incubated in 1 mL of medium containing breflidin A (10 µg/mL) in the absence or presence of HIV-1 Gag-peptide pools (15mers overlapping by 11 residues; NIH AIDS Research and Reference Reagent Program) or 1 µg/mL SEB (Sigma) for 6 hours. Cells were surface stained, permeabilized (Cytofix/Cytoperm kit; BD Biosciences), and stained with anti-CD3, anti-IFN-γ, anti-IL-2, anti-IL-17A, anti-IL-21 and anti-CD154. Events were collected on a modified LSRII flow cytometer (BD Immunocytometry Systems) and electronic compensation was performed with antibody capture beads (BD Biosciences). Data were analyzed using FlowJo Version 9.6 (TreeStar).

T and B cell culture

Co-culture experiments were performed with freshly isolated PBMC. 5×10^4 CD4 T cell populations were sorted based on expression of CCR7, CXCR5, CD150, CCR6 and PD-1 and cultured with 5×10^4 autologous naïve B cells (1:1 ratio) in the presence of SEB (0.5 µg/mL). Supernatants harvested on Day 2 were analyzed for cytokines using Luminex technology (Milliplex MAP Kit, HTH17MAG-14K, Millipore). The lower limit of detection (LOD) was set at the lowest concentration on the standard curve and values below the LOD were counted as zero. Supernatants collected on Day 12 were analyzed for immunoglobulins (Milliplex MAP Kit, HGAMMAG-301K). Some supernatants exceeded the saturation limit of the standard curves for IgM and IgG3. These values were included in the analysis and quantified as being equivalent to the highest determined concentration.

ELISA

Soluble CD14 and CXCL-13 (R&D Systems) were measured in plasma or sera from HIV-infected patients according to the manufacturer’s instructions.

CXCL-13 treatment

Freshly isolated PBMCs were incubated with recombinant human CXCL-13 (R&D Systems) at 37°C or 4°C and analyzed for CXCR5 surface expression by FACS.

Illumina deep sequencing of messenger RNA

CD4 T cell populations were sorted from uninfected PBMC (n = 5), HIV-infected PBMC (n = 5) and uninfected human tonsils (n = 4) based on expression of CCR7, CXCR5, CD150, CCR6 and PD-1 for PBMC and CD57, PD-1, CCR7, CXCR5, CCR4 and CXCR3 for tonsils. Total RNA was purified from sorted cell populations and treated with DNase I (Ambion) to minimize genomic DNA contamination. Polyadenylated RNA was isolated using Oligo-dT Dynabeads (Life Technologies), chemically fragmented, and used to construct barcoded Illumina Truseq libraries. Libraries were size-selected, quantified, pooled, size-selected and quantified again, and clustered on an Illumina Truseq Paired-End Flowcell v3. The flowcell was sequenced on an Illumina HiSeq 2000 in a 2×75-base paired-end, indexed run. Adaptor sequence was trimmed from the raw sequencing reads using Trimmomatic. The trimmed sequencing reads were subsequently aligned to the human genome (hg19) using TopHat. Differential expression testing was done using Cufflinks 2 and visualization of differential expression was done using the R package cummerbund. Accession numbers of the selected genes are shown in Supporting Table S2.

Virus neutralization

Neutralization activity of patient sera was determined against 20 viral isolates using a TZM-bl neutralization assay as previously described [20].

In vitro infection

Freshly isolated PBMCs were stimulated with PHA (10 µg/mL). After 12 hours stimulation, CXCR5high cells were sorted by FACS Aria based on surface molecule expression and infected by a multiplicity of infection (MOI) of 0.01 with either HIV NL-E or HIV NLAD8-E [41]. The infected cells were cultured in the presence of 50 U/mL recombinant human interleukin-2 (R&D) for 5 days and analyzed for CXCR5 expression by FACS.

Statistics

Experimental variables were analyzed using the nonparametric Mann-Whitney U test, the Wilcoxon matched-pairs signed rank test or the Friedman test with Dunn’s multiple comparison post-test. Correlation analysis was performed using the nonparametric
Spearman test. Error bars depict mean±SEM in all bar graphs shown. The GraphPad Prism statistical analysis program (GraphPad Software, version 5.0) was used throughout.

Supporting Information

Figure S1 Characterization of peripheral Tfh cells. (A) Scatter plot depicting frequency of IgG+ and IgA+ B cells in total B (CD19+) or naive B cells (CD19+CD27lowIgDhigh or CD19hiCD27lowIgDlow) for HIV-uninfected (n = 5), HIV-infected (non-viremic; n = 7) and HIV-infected (viremic; n = 11) donors. Both surface and intracellular staining of IgA and IgG were used to determine frequency. (B) Left: Representative flow cytometry plots from HIV-uninfected PBMC showing CXCR3 and CCR6 expression within CXCR5hi and CXCR5lo CD4 T cell subsets. Right: Scatter plot comparing the frequency of CXCR3 and CCR6 subsets within the CXCR5hi population from HIV-uninfected (open circles; n = 4) and HIV-infected individuals (closed circles; n = 8). (EPS)

Figure S2 Decrease of pTfh cells in HIV infection. (A) Pooled data showing the frequency (%) of indicated populations in CXCR5-expressing cells from PBMC from HIV-uninfected (open circles; n = 13), HIV-infected (treatment-naïve), CD4 count >200 (light gray circles; n = 44), and HIV-infected (treatment-naïve), CD4 count <200 (black circles; n = 22). Significant differences between uninfected and HIV-infected subjects were determined using the Mann-Whitney U test. **p<0.01; ***p<0.001; *p<0.05. Far Right: Correlative analysis between the frequency of CXCR5hiPD-1hi cells and the concentration of soluble CD14 in the sera or plasma. The trend did not reach statistical significance as determined by the Spearman test. (B) Top: Longitudinal analysis showing the frequency (%) of indicated populations in CXCR5-expressing cells from HIV-infected (treatment naïve) subjects (n = 10) over 36–48 months. No significant correlations were found. Bottom: Longitudinal analysis showing CD4 counts and viral loads from HIV-infected (treatment naïve) subjects (n = 10) over 36–48 months. (C) Pooled data showing the frequency (%) of indicated populations in CXCR5-expressing cells from PBMC from HIV-uninfected subjects (open circles; n = 13) and HIV-infected subjects before (n = 14, week 0; black circles) and after ART (week 24, dark gray circles; week 48, light gray circles). (EPS)

Figure S3 Characterization of pTfh cells in HIV infection. (A) CCR7hiCXCR3low and CCR7hiCXCR5lowCCR6hi CD4 T cells isolated from PBMCs were cultured with autologous naive B cells (CD19hiCD27lowIgD+). In the presence of SEB for 2 days and cytokine concentrations were measured from supernatants (HIV-uninfected, n = 5; HIV-infected (non-viremic), n = 4; HIV-infected (viremic), n = 0–1). Due to limited cell numbers we were unable to collect CCR7hiCXCR5loCCR6hi cells from viremic individuals. (B) Sorted CXCR5hi central memory cells isolated from blood do not down-regulate surface expression of CXCR5 upon X4 or R5 in vitro infection. (EPS)

Table S1 CD4 count, viral load and neutralization activity of subjects studied. (EPS)

Table S2 Accession numbers of selected genes. (EPS)

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Author Contributions

Conceived and designed the experiments: KLB RP TY CP RAK. Performed the experiments: KLB RP EB DA TY KW AW SN CP. Analyzed the data: KLB RP EB TY SD KW SN CP. Contributed reagents/materials/analysis tools: AM MR RH MC JA DCD JK. Wrote the paper: KLB RP TY CP RAK.

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