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Research Article

RESEARCH ARTICLE

Association of circulatory Tfh-like cells with neutralizing antibody responses among chronic HIV-1 subtype C infected long-term nonprogressors and progressors

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One sentence summary: This study addresses the correlation between Tfh subsets and breadth of neutralization in progressors and non-progressors HIV-infected individuals, showing a positive correlation between CXCR3+ Tfh cells and broadly neutralizing anti-HIV antibodies.

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ABSTRACT

HIV-1 vaccine functioning relies on successful induction of broadly neutralizing antibodies (bNAbs). CXCR3− circulatory T-follicular helper (cTfh) cells are necessary for inducing B-cells for generating bNAbs. Recent studies have suggested that CXCR3+ Tfh cells might also influence bNAb production. Plasma samples from 34 ART-Naïve HIV-1 infected individuals [long-term nonprogressors (LTNP)—19; Progressors—13] were tested against a heterologous virus panel ($n = 11$) from

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subtypes A, B, C, G, AC, BC and AE. Frequencies of CXCR3+ and CXCR3− cTfh-like cells in peripheral circulation were studied using flow cytometry. LTNP showed significantly lower CXCR3+ and higher CXCR3− cTfh-like cell frequencies, while neutralization breadth was observed to be broader in progressors. A positive correlation was observed between bNAb breadth and potency with CXCR3+PD-1+ cTfh-like cells in LTNP. Based on neutralization breadth, 9 HIV-1 infected individuals were classified as 'top neutralizers' and 23 as 'low neutralizers' and they did not show any correlations with CXCR3+ and CXCR3− cTfh-like cells. These preliminary data suggest that CXCR3+ similar to CXCR3− might possess significant functional properties for driving B-cells to produce bNAbs. Hence, an HIV vaccine which is capable of optimal induction of CXCR3+ cTfh cells at germinal centers might confer superior protection against HIV.

Keywords: circulatory Tfh-like cells; broadly neutralizing antibodies; MPER binding antibodies; HIV-1 neutralizers

INTRODUCTION

Neutralizing antibodies are the principal correlates of protection for most licensed preventive vaccines (Leo, Cunningham and Stern 2011). Generation of broadly neutralizing antibodies (bNAbs) by B-cells requires specialized CD4+ T-cells called T-follicular helper (Tfh) cells, which are critical for the priming of B-cell responses in lymph node germinal centers (GC), thus leading to the development of bNAbs (Crotty 2014). Tfh cells induce activation and differentiation of B-cells into immunoglobulin (Ig) secreting cells, Ig class switching and affinity maturation in B-cells (Ma and Deenick 2014; Sage and Sharpe 2015; Avery *et al.* 2008). Tfh cells express IL-21 that helps GC B-cells to proliferate and differentiate into plasma cells resulting in production of efficient and high affinity antibodies (Vinuesa *et al.* 2016; Thornhill *et al.* 2017).

In natural infection, ∼10 to 20% of HIV-1 infected individuals develop bNAbs, ∼2–4 years post infection. These bNAbs are characterized by extensive somatic hyper mutation within the long heavy-chain CDR3 (HCDR3) regions and are induced by memory B-cells. Impaired generation of memory B-cells might be a result of deteriorated CD4+ T-cell compartment, especially Tfh cells, that becomes qualitatively incapable of providing adequate help to B-cells during the course of HIV infection (Ho *et al.* 2011; McHeyzer-Williams *et al.* 2012). Hence, optimum induction of B-cells to generate bNAbs against the HIV-1 envelope (env) is a key goal in HIV-1 vaccine development. Development of neutralization breadth has been associated with high viral load, low CD4+ T-cell counts, viral evolution and antibody maturation (Moore, Williamson and Morris 2015; Landais *et al.* 2016). It is likely that antigen load and virus evolution provide the basis for the process of antibody maturation (Bhiman *et al.* 2015), yet despite high levels of virus, majority of HIV-1 infected individuals do not develop broadly neutralizing antibodies (Gray *et al.* 2011). Since it is relatively difficult to study and understand GC Tfh cell function due to inaccessibility and paucity of specimens from lymph nodes, a memory Tfh-like population of CD4+CXCR5+ T cells has been identified in the peripheral circulation which share similar functional properties with GC Tfh cells (Morita *et al.* 2011; Lu *et al.* 2018).

It is well established that antibody responses induced by antigens after a single or few exposures depend on the generation of plasma cells and memory B-cells within GCs of secondary lymphoid organs. In the GC, Tfh cells, which express high levels of CXCR5, provide critical support for the differentiation of B-cells into affinity-matured plasma cells and memory B-cells through their production of cytokines such as IL-4 and IL-21 and co-stimulatory molecules such as CD40L. After providing help to B-cells, Tfh cells become memory CXCR5+CD4+ Tfh cells that recirculate in blood and then return to the GC upon antigen re-exposure. These peripheral memory Tfh cells have been further subdivided into distinct subsets based on expression of CXCR3 and CCR6 receptors. However, the role of CXCR5+CXCR3+PD-1+Tfh cells and CXCR5+CXCR3−PD-1+ Tfh cells in the development of humoral immunity and antibody responses is dependent on the dynamics of disease progression, immune activation and overall viremia of the infected individual and remains a case for further investigation (Martin-Gayo *et al.* 2017). It is reported that there exists an association between the frequencies of circulating CXCR5+CXCR3–PD-1+ Tfh cells and the development of neutralizing antibodies among HIV-1 infected individuals with constantly high plasma viral loads and high immune activation (Locci *et al.* 2013). In contrast CXCR3+CXCR5+PD-1+CD4+ T-cell frequencies correlated in individuals immunized with influenza vaccine (Bentebibel *et al.* 2013) and HPV vaccine (Matsui *et al.* 2015). CXCR3+CXCR5+CD4+ T cells frequencies were also reported in blood and lymph nodes of SIV vaccinated rhesus macaques (Iyer *et al.* 2015). A recent study also reported induction of CXCR3+Tfh in chronic SIV infections (Velu *et al.* 2016). The current study investigates the relationship between peripheral Tfh-like cell frequencies and neutralizing antibody responses in the context of controlled HIV infection.

MATERIALS AND METHODS

Subjects and sample collection

In this cross-sectional study, HIV-1 subtype C infected individuals attending YRG CARE medical center were screened based on their CD4+ T-cell counts and length of HIV infection. Samples from a cohort of long-term nonprogressors (LTNP) (n = 19), defined as individuals who maintained peripheral CD4+ T-cell counts of >500 cells/mm^3 for >7 years in the absence of ART, and progressors (n = 13), defined as individuals with a declining CD4+ T-cell count but who remained at cell counts of 300–500 cells/mm^3 3–5 years post infection without receiving ART were used for this study. All progressors initiated ART within 9 months of the initial sample collection visit. This study was approved by the YRG CARE institutional review board and all volunteers provided written informed consent. Demographics and clinical characteristics of study participants are summarized in Table 1.

According to standard procedures, peripheral blood mononuclear cells (PBMCs) were harvested from EDTA-treated peripheral blood using Ficoll-Paque density gradient centrifugation method and cryopreserved at ≤−140°C until testing. Blood specimens were also collected in Becton Dickinson (BD) serum separating tube and centrifuged at 3000 rpm for 10 minutes. Serum was separated and stored at −70°C until testing.

Plasma viral load and CD4+ T-cell count

Absolute CD4+ T-cell counts were tested using FACS count (BD Biosciences, San Jose, CA, USA). Plasma HIV-1 viral load (PVL)

Table 1. Demographic characteristics of study participants.

Factors	LTNP (n = 19)	Typical Progressors (n = 13)	P-value
Age (IQR)	34 (30,39)	36 (32,40)	0.5552
Median CD4 T-cell count (IQR)	752 (612.3947)	384 (318,416)	**<0.05**
Median Plasma viral load (IQR)	1319.5 (133,12 917)	113 939 (24 107,210 389)	**<0.05**
Median CD8 T-cell count (IQR)	1121 (764,1371)	998.5 (808.8,1326)	0.1556
Median CD4% (IQR)	27 (21,36)	20 (14,22)	**0.001**
Median CD8% (IQR)	49 (37.5,59.5)	55 (45,61)	0.173
Median CD4/CD8 ratio (IQR)	0.66 (0.495,0.95)	0.35 (0.25,0.44)	**0.003**
Median Total WBC count (IQR)	7.55 (7.05,8)	6.3 (5.7,7.4)	**0.028**
Median TLC (IQR)	2.75 (2.25,3.2)	2 (1.5,2.7)	**0.018**
Media lymphocyte count (IQR)	36.55 (28.68,43.4)	35.3 (26.3,38)	0.429

were measured using m2000rt Abbott Real-Time PCR (Abbott Molecular Inc., IL, USA).

Immunofluorescence staining and flow cytometric analysis

Cryopreserved PBMCs were thawed and rested overnight in Rosewell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum. Cells were then washed, counted and stained with live/dead fixable blue dead cell stain (L23105, ThermoFisher, MA, USA) for live/dead discrimination and incubated in dark for 20 minutes, prior to staining with fluorescent antibodies. Following incubation, cells were washed and stained with following fluorescent antibodies: anti-CD3 APC-H7 (clone SK7; BD Pharmingen, CA, USA), anti-CD4 PerCPCy5.5 (clone RPA-T4; BD Pharmingen, CA, USA), anti-CXCR5 BB515 (clone RF8B2; BD Horizon, CA, USA), anti-PD-1 PE (clone EH12.1; BD Pharmingen, CA, USA) and anti-CXCR3 BUV395 (clone IC6; BD Horizon, CA, USA). Following incubation, cells were washed, fixed and acquired using a FACS Aria flow cytometer (BD Biosciences, MD, USA). Data were analyzed using Flow Jo v10.1r5 (Tree Star Inc., OR, USA). Fluorescence minus one (FMO) controls were used to determine the gates of each critical antibody markers (CXCR5, CXCR3 and PD-1) analyzed in this study. CD4+CXCR5+ T-cells were identified, which are further gated based on the expression of CXCR3 and PD-1 to study the frequencies of CXCR3+PD-1+cTfh-like cells and CXCR3−PD-1+ cTfh-like cells. Representative flow cytometry gating strategy for identifying cTfh-like cells is shown in Figure S1 (Supporting Information).

Statistical analysis

Mann–Whitney U test was performed to derive the significance between the groups. Spearman's rank correlations were used to examine bivariate associations between study variables and a P-value of <0.05 was considered significant. Fisher's exact test two-tailed was used to compare categorical variables and one-way ANOVA was performed to compare more than two groups. All statistical analyses were performed using GraphPad Prism version 5.0 software (GraphPad Software Inc., CA, USA).

HIV-1 pseudovirus production and TCID titration

HIV-1 pseudoviruses were prepared by co-transfection of envelope expressing plasmid with *env*deleted HIV-1 backbone plasmid (pSG3ΔEnv) (Montefiori 2005). In brief, vectors were co-transfected into 293T cells in 6-well tissue culture plates using the FuGENE6 Transfection kit (Promega Inc., WI, USA). Cell supernatants containing pseudoviruses were harvested 48 hours post-transfection, aliquoted, and stored at −80°C. Tissue culture infective dose (TCID) assays were performed using TZM-bl cells (1×10^5 cells/mL) containing DEAE-Dextran (25 μg/mL) in 96-well microtiter plates. Infectivity titers were determined by measuring luciferase activity using Britelite luciferase substrate (Perkin Elmer Inc., MA, USA) with a Victor X3 Luminometer (Perkin Elmer Inc., MA, USA).

HIV-1 neutralization assay

The measurement of antibody-dependent HIV-1 neutralization was determined as a function of reduction in luciferase reporter gene expression after a single round of infection in TZM-bl (Montefiori 2005). Briefly, patient plasma was heat inactivated at 56°C for 30 minutes. Plasma samples were 3-fold serially diluted with a starting dilution of 1:20 in Dulbecco-modified Eagle medium (Gibco, CA, USA) containing 10% fetal bovine plasma (Hyclone Laboratories, UT, USA). Each pseudovirus preparation was pretitrated to determine the optimal TCID dilution using TZM-bl cells. Then, 50 μL of *env* pseudovirus was added and kept for 45 to 90 minutes at 37°C in 5% CO_2. TZM-bl cell monolayer was trypsinized after 1 hour and 10 000 cells were seeded per well in 100 μL of growth media and incubated for 48 hours at 37°C in 5% CO_2. A virus control (pseudovirus and cells) and a cell control well were assigned for each assay plate. After 48-hour incubation, 150 μL of supernatant was discarded from each well and 100 μL of substrate (BriteLite plus Reporter Gene Assay System, PerkinElmer Life and Analytical Sciences, CT, USA) was added to each well for 2 minutes. 150 μL of lysed suspension was transferred into a 96-well black solid plate (Costar Corning, NY, USA) for measurements of relative luminescence units (RLU) using the Victor X3 Luminometer Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences, Turku, Finland).

Neutralizing antibody titers were expressed as the reciprocal of the plasma dilution required to reduce RLU by 50% compared to those of the pseudovirus control wells after subtraction of background RLUs. 50% inhibitory dilutions (ID_{50}) were calculated based on a curve fit to each neutralization assay using the Atlas Macro online software tool. Study specimens were assayed against a standard murine leukemia virus (MLV; were kindly provided by Dr David C. Montefiori, Duke University Medical Center, Durham, NC, USA) containing pSG3D *env* backbone plasmid to discount the presence of antiretroviral (ARV) drugs. Any neutralizing activity against these MLV was due to the presence of ARV drugs in the specimens.

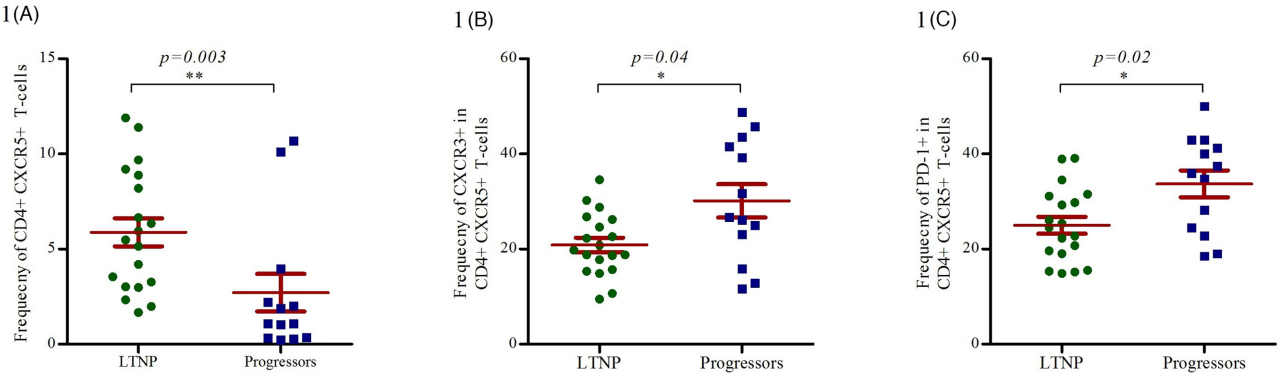

Figure 1. Graphical plot showing the differences in frequencies of various CD4+CXCR5+ T-cell subsets between LTNP and progressors **(A)** frequency of CD4+CXCR5+ T-cells, **(B)** frequency of CXCR3 expressing CD4+CXCR5+ T-cells and **(C)** frequency of PD-1 expressing CD4+CXCR5+ T-cells.

Reagents

Molecularly cloned *env* pseudoviruses used in this study were obtained from Dr Hagen von Briesen, Fraunhofer Institutfür Biomedizinische Technik IBMT, Sulzbach, Germany and Dr David Montefiori, Laboratory for AIDS Vaccine Research & Development, Duke University Medical Center, Durham, USA. TZM-bl indicator cells were obtained from Drs John Kappes and Xiaoyun Wu through the 'NIH AIDS Reagent Program' (NIH ARP). Human anti-gp120 carbohydrate specific mAb 2G12, human anti gp41 monoclonal antibody (mAb) 2F5 and 4E10, recombinant sCD4 and HIV IgG were kindly provided by Dr David C. Montefiori, Duke University Medical Center, Durham, NC, USA.

RESULTS

Characterization of CD4+ T-cells. The frequencies of CD4+CXCR5+ T-cells (CXCR5+ T-cells gated from CD4+ T-cells) were analyzed, before characterizing the rare subpopulation of cTfh-like cells. It was observed that CD4+CXCR5+ T-cells were significantly higher in LTNP than in progressors (Fig. 1a; $P = 0.003$). Further characterization of CD4+CXCR5+ T-cells showed that LTNP had significantly lower frequencies of CXCR3 expressing CD4+CXCR5+ T-cells (Fig. 1b; $P = 0.04$) and PD-1 expressing CD4+CXCR5+ T-cells (Fig. 1c; $P = 0.02$). Absolute counts of all the above cell populations were observed to be significantly higher in LTNP compared to progressors (Figure S2, Supporting Information). Except PD-1+ T-cells gated from CD4+CXCR5+ T-cells of LTNP showing significant correlation with PVL ($r = 0.52$; $P = 0.02$), none of the above analyzed cell populations showed correlation with either CD4+ T-cell counts or PVL in both LTNP and progressors.

Frequencies of cTfh-like cells in LTNP and progressors

LTNP had significantly lower frequencies of CXCR3+PD-1+ cTfh-like cells compared to progressors (Fig. 2a; $P = 0.002$). When the LTNP cohort was further stratified based on their PVL as low viremic LTNP (PVL < 2000 copies/mL) and high viremic LTNP (PVL > 2000 copies/mL), significant lower frequencies of CXCR3+PD-1+ cTfh-like cells were seen in low viremic LTNP ($P < 0.05$) as well as high viremic LTNP ($P < 0.05$), compared to progressors (Fig. 2b). When CXCR3−PD-1+ cTfh-like cells were taken into account, no significant differences were found in frequencies of CXCR3−PD-1+ cTfh-like cells between LTNP and progressors (Fig. 2c; $P = 0.19$). PVL stratification also showed no significant differences in CXCR3− PD-1+ cTfh-like cells frequencies between low viremic LTNP vs high viremic LTNP vs progressors (Fig. 2d).

Double negative CXCR3−PD-1− cTfh-like cell population was observed to be significantly higher in LTNP compared to progressors (Fig. 2e; $P = 0.02$). PVL grouping of study population showed that low viremic LTNP had significantly higher CXCR3−PD-1− cTfh-like cells than progressors ($P < 0.05$) whereas, high viremic LTNP and progressors comparison showed no significant differences (Fig. 2f). No significant differences were found between LTNP and progressors when CXCR3+PD-1^{low} (Fig. 2g) and CXCR3+PD-$1+^{high}$ (Fig. 2h) cTfh-like cells were compared. There were no significant correlations seen, when CXCR3+PD-1+ cTfh-like cells and CXCR3−PD-1+ cTfh-like of LTNP and progressors compared to their respective CD4+ T-cell count and PVL (Table 2). LTNP had higher absolute counts of all the above cTfh-like cell populations compared to progressors (Figure S3, Supporting Information).

HIV-1 progressors demonstrated greater neutralization ability (breadth and potency) than LTNP volunteers

Thirty-two plasma samples from long term nonprogressors ($n = 19$) and progressors ($n = 13$) were screened against a heterologous global virus panel ($n = 11$) including subtype A, B, C, G, AC, BC & AE viral isolates (Table 3A). The neutralization breadth of a specimen was defined as the number of *env* pseudoviruses neutralized with an ID_{50} titer > 100. The neutralization potency of a specimen was defined as the ID_{50} geometric mean titer (GMT) obtained against the tested pseudovirus panel. Based on the neutralization breadth, both the study cohorts were grouped and divided into two categories: 'low neutralizers' whose plasma samples neutralized < 50% of viruses tested and 'top neutralizers' whose plasma samples neutralized > 50% of viruses tested (Table 3B). 'Elite neutralizers' were termed as plasma samples that are able to neutralize > 75% of viruses tested with geometric mean ID_{50} > 500. A total of 28% (9/32) of volunteers were categorized as top neutralizers. One sample (NTP012) neutralized the entire set of viruses tested, and was classified as an elite neutralizer. Neutralization breadth (Fig. 3a, $P = 0.005$) and potency (Fig. 3b, $P = 0.0008$) was significantly higher among progressors [Breadth 50 (29–73); GMT 109 (64–177)] than LTNP volunteers [(Breadth 18 (9–27); GMT 46 (36–57)].

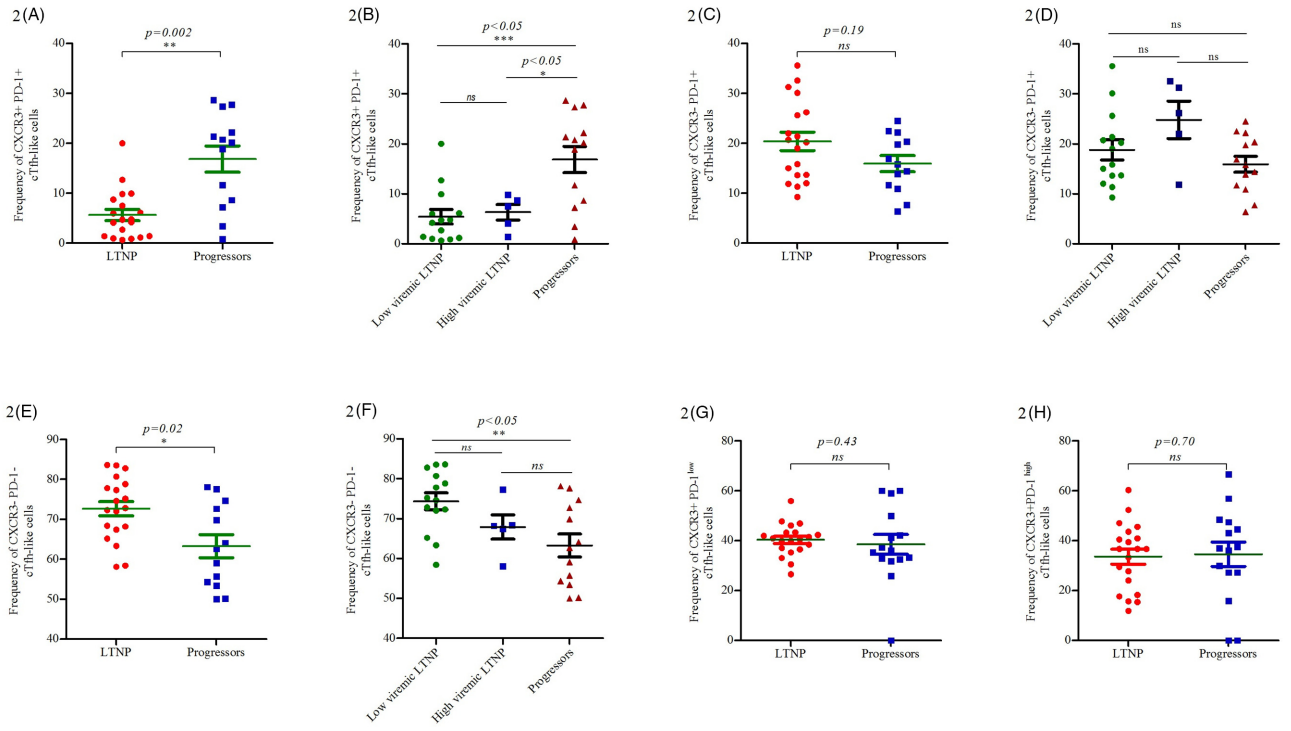

Figure 2. Graphical plot showing the differences in frequencies of various cTfh-like populations. **(A)** CXCR3+PD-1+ cTfh-like cells between LTNP and progressors, **(B)** ANOVA plot showing differences in CXCR3+PD-1+ cTfh-like cells between low viremic LTNP, high viremic LTNP and progressors **(C)**, CXCR3−PD-1+ cTfh-like cells between LTNP and progressors **(D)**, ANOVA plot showing differences in CXCR3−PD-1+ cTfh-like cells between low viremic LTNP, high viremic LTNP and progressors **(E)**, CXCR3−PD-1− cTfh-like cells between LTNP and progressors, **(F)** ANOVA plot showing differences in CXCR3−PD-1− cTfh-like cells between low viremic LTNP, high viremic LTNP and progressors, **(G)** CXCR3+PD-1+^{low} between LTNP and progressors and **(H)** CXCR3+PD-1+^{high} between LTNP and progressors.

Table 2. Relationship of CXCR3+PD-1+ and CXCR3−PD-1+ cTfh-like with CD4+ T-cell counts and PVL.

	LTNP				Progressors			
	CD4+ T-cell counts		PVL		CD4+ T-cell counts		PVL	
cTfh-like cells	Spearman *r*	*P*-value	Spearman *r*	*P*-value	Spearman *r*	*P*-value	Spearman *r*	*P*-value
CXCR3+PD-1+ cTfh-like cells	−0.38	0.10	0.36	0.13	−0.12	0.71	0.14	0.64
CXCR3−PD-1+ cTfh-like cells	−0.13	0.59	0.32	0.17	0.07	0.80	0.15	0.42

Correlations of cTfh-like cells with HIV-1 neutralization

In LTNP, the frequency of CXCR3+PD-1+ cTfh-like cells positively correlates with neutralization breadth (Fig. 4a; $r = 0.52$, $P = 0.02$) and potency (Fig. 4b; $r = 0.63$, $P = 0.003$) while no such significant correlations were observed in progressors in both neutralization breadth (Fig. 4c; $r = 0.24$, $P = 0.41$) and potency (Fig. 4d; $r = 0.31$, $P = 0.29$). A notable negative correlation was observed between CXCR3− PD-1+ cTfh-like cells with neutralization breadth (Fig. 4e; $r = -0.40$, $P = 0.09$) and potency (Fig. 4f; $r = -0.34$, $P = 0.14$) in LTNP. In progressors, neither neutralization breadth (Fig. 4g; $r = -0.16$, $P = 0.60$) nor potency (Fig. 4h; $r = -0.07$, $P = 0.83$) exhibited any correlation with CXCR3−PD-1+ cTfh-like cells frequencies. No meaningful correlations were observed between the frequency of CXCR3−PD-1− cTfh-like cells and neutralization breadth as well as potency in both LTNP and progressors. Strong positive correlations were observed when overall CXCR3+PD-1+ cTfh-like cells were compared with neutralization breadth (Fig. 4i; $r = 0.66$, $P < 0.0001$) and potency (Fig. 4j; $r = 0.70$, $P < 0.0001$), irrespective of the study cohort. Overall, CXCR3−PD-1+ cTfh-like cells frequencies showed significant negative correlation with neutralization breadth (Fig. 4k; $r = -0.37$, $P = 0.04$) with neutralization potency showing no correlation (Fig. 4l; $r = -0.33$, $P = 0.06$). Based on the neutralization capability, both LTNP and progressor cohorts were categorized into top neutralizers and low neutralizers and analyzed. No significant differences were found between top neutralizers and low neutralizers in both CXCR3+PD-1+ (Fig. 5a; $P = 0.06$) and CXCR3− PD-1+ (Fig. 5b; $P = 0.42$) cTfh-like cell frequencies. When neutralization breadth was compared, top neutralizers showed no correlations with both CXCR3+PD-1+ (Fig. 5c; $r = 0.26$, $P = 0.49$) and CXCR3−PD-1+ (Fig. 5d; $r = -0.008$, $P = 0.98$) cTfh-like cells. Neutralization breadth of low neutralizers showed significant positive correlation with CXCR3+PD-1+ cTfh-like cells (Fig. 5e; $r = 0.59$, $P = 0.003$) and significant negative correlation with CXCR3−PD-1+ cTfh-like cells (Fig. 5f; $r = -0.48$, $P = 0.02$).

Table 3A. Neutralization Titers (ID 50) of plasma samples from LTNP ($n = 20$) against Global Virus panel (Subtype A, B, C, G, AC, BC and AE).

Patient ID	CD4 count (Cells/μL)	Viral load (Copies/mL)	HIV-1 Q259.d12.17 A—Kenya	HIV-1 TRO11 B—Italy	HIV-1 16 936–2.21 C—India	HIV-1 16 055–2.3 C—India	HIV-1 ZM249.PL1 C—Zambia	HIV-1 DU156.d12 C—S. Africa	HIV-1 px1632 G—Spain	HIV-1 246F3 AC- Tanzania	HIV-1 CH119.10 BC—China	HIV-1 CNE8 AE—China	HIV-1 CNE55 AE—China	Neutralization potency (GMT ID_{50} Titer)	Neutralization breadth (%)
LTNP 03	785	150	408	230	227	93	99	115	53	53	37	57	171	108	45
LTNP 07	703	15 799	20	65	22	100	21	64	20	24	20	38	592	43	9
LTNP 08	1164	150	20	21	20	20	20	29	20	20	20	112	26	25	9
LTNP 09	1008	154	20	26	20	20	20	35	20	24	20	242	144	33	18
LTNP 11	966	5803	20	65	20	20	20	63	20	20	20	33	218	32	9
LTNP 13	905	40	20	126	20	44	135	87	22	34	20	98	259	53	27
LTNP 14	500	1495	20	63	20	20	20	45	31	36	20	55	225	36	9
LTNp 15	1044	40	20	87	20	20	20	35	30	32	20	59	179	35	9
LTNP 17	808	370	20	163	20	20	20	215	24	27	20	56	256	43	27
LTNP 18	870	82	20	33	20	20	62	36	20	29	20	48	60	30	0
LTNP 19	719	40	20	89	20	20	48	90	20	20	53	50	1018	48	9
LTNP 20	947	1839	20	20	20	20	20	26	20	63	26	144	687	38	18
LTNP 21	630	40	20	99	20	20	20	20	20	26	20	46	1048	37	9
LTNP 22	1055	1144	31	216	20	20	20	61	20	40	20	82	725	48	18
LTNP 26	501	2259	20	928	152	20	90	269	123	24	46	221	220	99	55
LTNP24	558	10 785	59	90	25	91	74	138	20	109	53	87	34	61	18
LTNP27	566	3994	20	45	20	20	109	372	47	52	26	83	169	55	27
N-LTNP002	538	12 917	20	20	20	20	34	20	154	63	185	165	130	49	36
N-LTNP004	728	1332	20	1495	104	231	280	420	40	80	94	232	3214	199	64
N-LTNP005	762	1034	20	176	20	24	20	64	20	85	51	391	1715	67	27
			Neutralization Titers (ID_{50}) of plasma samples from Progressors ($n = 14$) against Global Virus panel (Subtype A, B, C, G, AC, BC & AE)												
NTP 004	289	22 989	20	73	164	20	342	175	20	45	36	286	344	80	45
NTP 006	398	24 107	174	773	129	247	152	272	20	96	20	114	589	142	73
NTP 007	416	351 000	20	262	169	24	262	192	82	50	244	57	64	92	45
NTP 008	456	22 821	20	38	92	31	20	44	51	116	47	594	108	60	27
NTP 011	384	337 650	116	226	66	137	520	393	59	218	229	765	81	185	73
NTP 012	358	647 625	8464	760	770	6002	345	183	442	456	438	594	1174	822	100
NTP 013	462	210 389	26	35	37	386	131	142	45	59	41	325	60	76	36
NTP 018	435	226 452	20	28	20	20	20	184	20	32	63	99	133	40	18
NTP 019	401	85 997	133	1226	53	116	242	151	94	29	339	71	380	151	64
NTP 020	392	11 072	48	175	56	51	110	227	20	78	257	494	1118	126	55
NTP 022	318	113 939	63	33	89	112	332	642	121	406	1215	829	1099	250	73
NTP 023	383	31 909	20	31	21	30	30	27	20	67	60	328	570	50	18
NTP 026	331	139 308	174	390	203	196	367	104	181	383	166	202	97	202	91
NTP 031	313	16 498	20	20	20	477	20	20	24	79	27	222	262	50	27

Table 3B. Categorization of 34 HIV Positive individuals by HIV-1 Neutralizing activity.

Low neutralizers			Top neutralizers		
Patient ID	Neutralization potency (GMT ID_{50} Titer)	Neutralisation breadth (%)	Patient ID	Neutralization potency (GMT ID_{50} Titer)	Neutralisation breadth (%)
LTNP 18	30	0	LTNP 26	99	55
LTNP 07	43	9	NTP 020	126	55
LTNP 08	25	9	N-LTNP004	199	64
LTNP 11	32	9	NTP 019	151	64
LTNP 14	36	9	NTP 006	142	73
LTNp 15	35	9	NTP 011	185	73
LTNP 19	48	9	NTP 022	250	73
LTNP 21	37	9	NTP 026	202	91
LTNP 09	33	18	NTP 012	822	100
LTNP 20	38	18			
LTNP 22	48	18			
LTNP24	61	18			
NTP 018	40	18			
NTP 023	50	18			
LTNP 13	53	27			
LTNP 17	43	27			
LTNP27	55	27			
N-LTNP005	67	27			
NTP 008	60	27			
NTP 031	50	27			
N-LTNP002	49	36			
NTP 013	76	36			
LTNP 03	108	45			
NTP 004	80	45			
NTP 007	92	45			

DISCUSSION

The vast majority of licensed vaccines work through the induction of an antibody response against a pathogen. In addition to a functional cytotoxic response T-cells, and the CD4+ T-cell subset, are also able to provide help to B-cells for the production of antibodies. Understanding the factors involved in the process by which CD4+ T-cell help is elicited remains vital to the development of an effective HIV vaccine (Streeck *et al.* 2013). The discovery and characterization of a specialized subset of CD4+ T-cells residing within the lymphoid follicle termed T follicular helper cells (Tfh), whose main function is to support B cell maturation and differentiation to produce high affinity antibodies (Vinuesa *et al.* 2016), was pivotal to this effort.

Studies have shown that frequency of Tfh cells increases despite CD4+ T-cell decline, and this increase in frequency of Tfh cells associates with increased antibody production (Hong *et al.* 2012; Cubas *et al.* 2013; Perreau *et al.* 2013; Mylvaganam *et al.* 2014). CXCR5+CD4+ T-cells present in peripheral circulation were shown to shape the quality of human humoral immunity (Morita *et al.* 2011). CD4+CXCR5+CXCR3−PD-1+ T-cells were the widely studied Tfh cells which are considered as the bonafide Tfh cell population in the peripheral circulation and reported to possess superior Tfh cell activity in helping B-cells to generate neutralizing antibodies (Locci *et al.* 2013; Ma and Deenick 2014; Obeng-Adjei *et al.* 2015). A strong correlation was observed between CD4+CXCR5+CXCR3−PD-1+ T-cells and HIV-1 specific bNAbs was observed in HIV patients with progressive disease (Locci *et al.* 2013).These studies indicate the frequencies of Tfh cells in conditions where there is progressive and active HIV replication with high T-cell activation rates. However, there have been studies on HIV-1 controller cohorts to study the HIV immune defense mechanism for studying HIV-1–specific antibodies with increased neutralizing breadth in the absence of high-levels of viremia. It was observed that ratios between CXCR5+CXCR3+PD-1^{lo} and CXCR3+CXCR5+$PD1^{hi}$ Tfh-like cells in blood from HIV-1 controllers correlated with increased neutralizing breadth of HIV-1antibodies. In contrast, peripheral CXCR5+CXCR3–CD4+ T cells were unrelated to HIV-1–specific antibodies with broader neutralizing breadth (Martin-Gayo *et al.* 2017). Thus, both CXCR3+ and CXCR3− receptors have been shown to induce neutralization response based on the dynamics of disease progression and present viremia. In the current context, we explore the dynamics of cTfh cells and their relationship with bNAbs in HIV infected people with low viremia who present no signs of progressive disease. Absolute counts of cTfh-like cells were observed to differ from the frequency comparisons since absolute count reflects exact measure of number of cells positive for cTfh-like cell markers from the gated lymphocytes whereas percentage expresses the relative measure from the gated lymphocytes.

Here, LTNP showed a significant increase in CD4 + CXCR5 + T-cell frequencies than progressors. It is observed that during early HIV infection CD4+CXCR5+ T-cells is preserved resulting in the development of bNAbs during chronic stages (Cohen *et al.* 2014). Though CD4+CXCR5+ T-cell population might have been preserved in the LTNP studied, lower level of bNAbs in LTNP should be due to inadequate antigenic stimulation required for the development of bNAbs (Nandagopal *et al.* 2018). However, significant conclusions can be arrived only by studying other B-cell inducing factors such as IL-21 and IL-4 which help B-cells to produce bNAbs, especially in viremic LTNP. Increase in CXCR3 expressing and PD-1 expressing T-cells gated from CD4+CXCR5+ T-cells were observed in progressors, in this study.

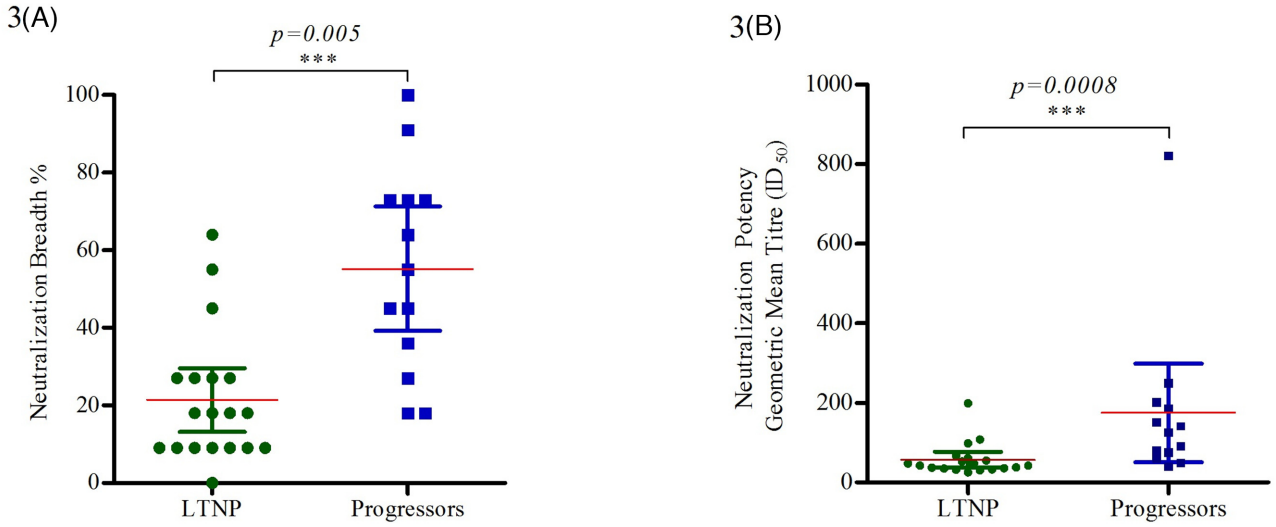

Figure 3. Graphical plot showing the comparison of HIV-1 **(A)** Neutralization Breadth **(B)** Neutralization Potency between LTNP and progressors. LTNP exhibit significantly lower breadth and potency of HIV-1 neutralization than progressors.

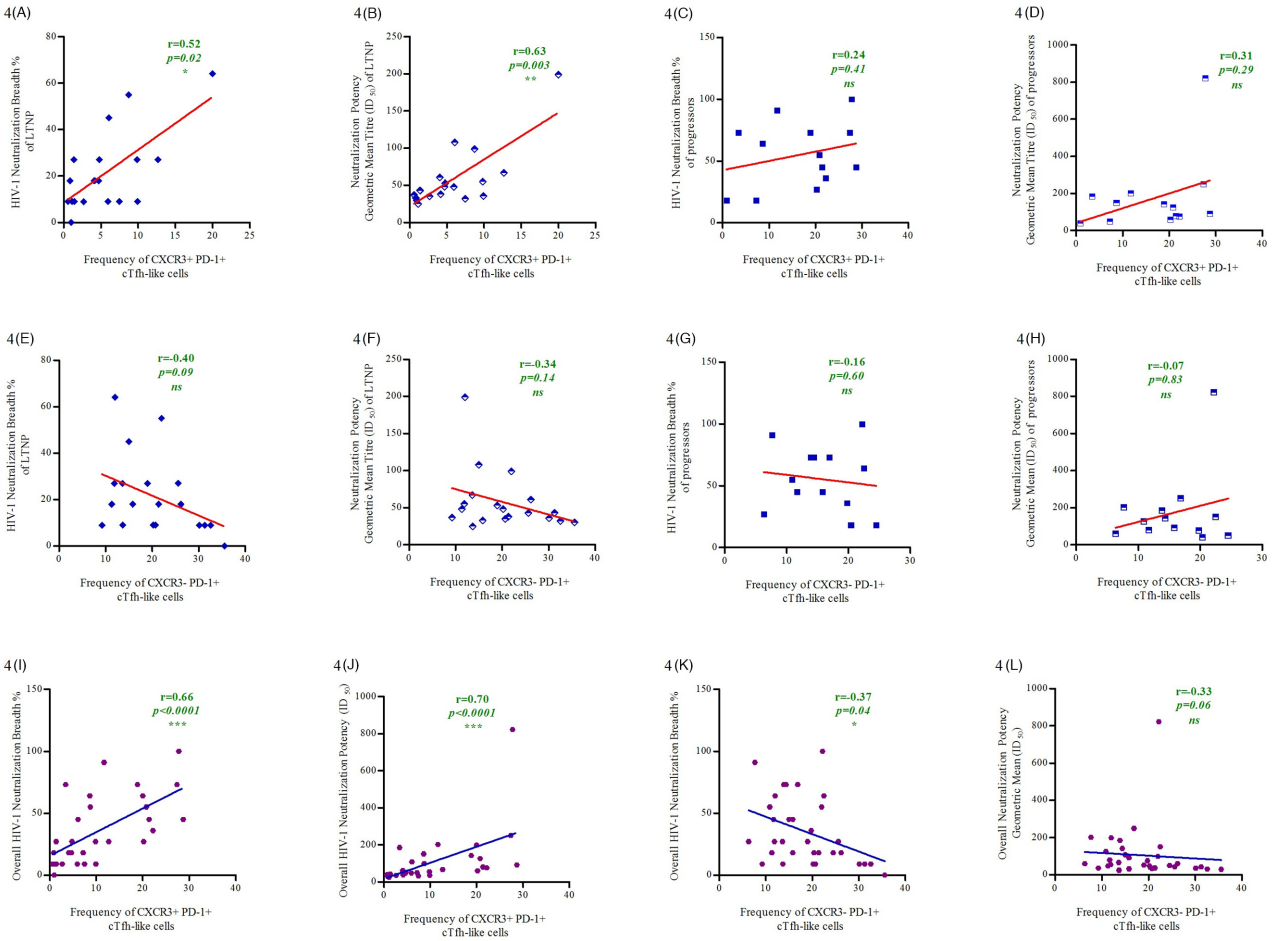

Figure 4. Graphical plot showing correlations of cTfh-like cell frequencies of LTNP and progressors with their respective neutralization breadth and potency, and overall (both LTNP and progressors) correlation between cTfh-like cells and neutralization breadth and potency **(A)** CXCR3+PD-1+ cTfh-like cells vs Neutralization Breadth of LTNP **(B)** CXCR3+PD-1+ cTfh-like cells vs Neutralization Potency of LTNP **(C)** CXCR3+PD-1+ cTfh-like cells vs Neutralization Breadth of progressors **(D)** CXCR3+PD-1+ cTfh-like cells vs Neutralization Potency of progressors **(E)** CXCR3−PD-1+ cTfh-like cells vs Neutralization Breadth of LTNP **(F)** CXCR3−PD-1+ cTfh-like cells vs Neutralization Potency of LTNP **(G)** CXCR3−PD-1+ cTfh-like cells vs Neutralization Breadth of progressors **(H)** CXCR3−PD-1+ cTfh-like cells vs Neutralization Potency of progressors **(I)** overall CXCR3+PD-1+ cTfh-like cells vs Neutralization Breadth **(J)** overall CXCR3+PD-1+ cTfh-like cells vs Neutralization Potency **(K)** overall CXCR3−PD-1+ cTfh-like cells vs Neutralization Breadth and **(L)** overall CXCR3−PD-1+ cTfh-like cells vs Neutralization Potency.

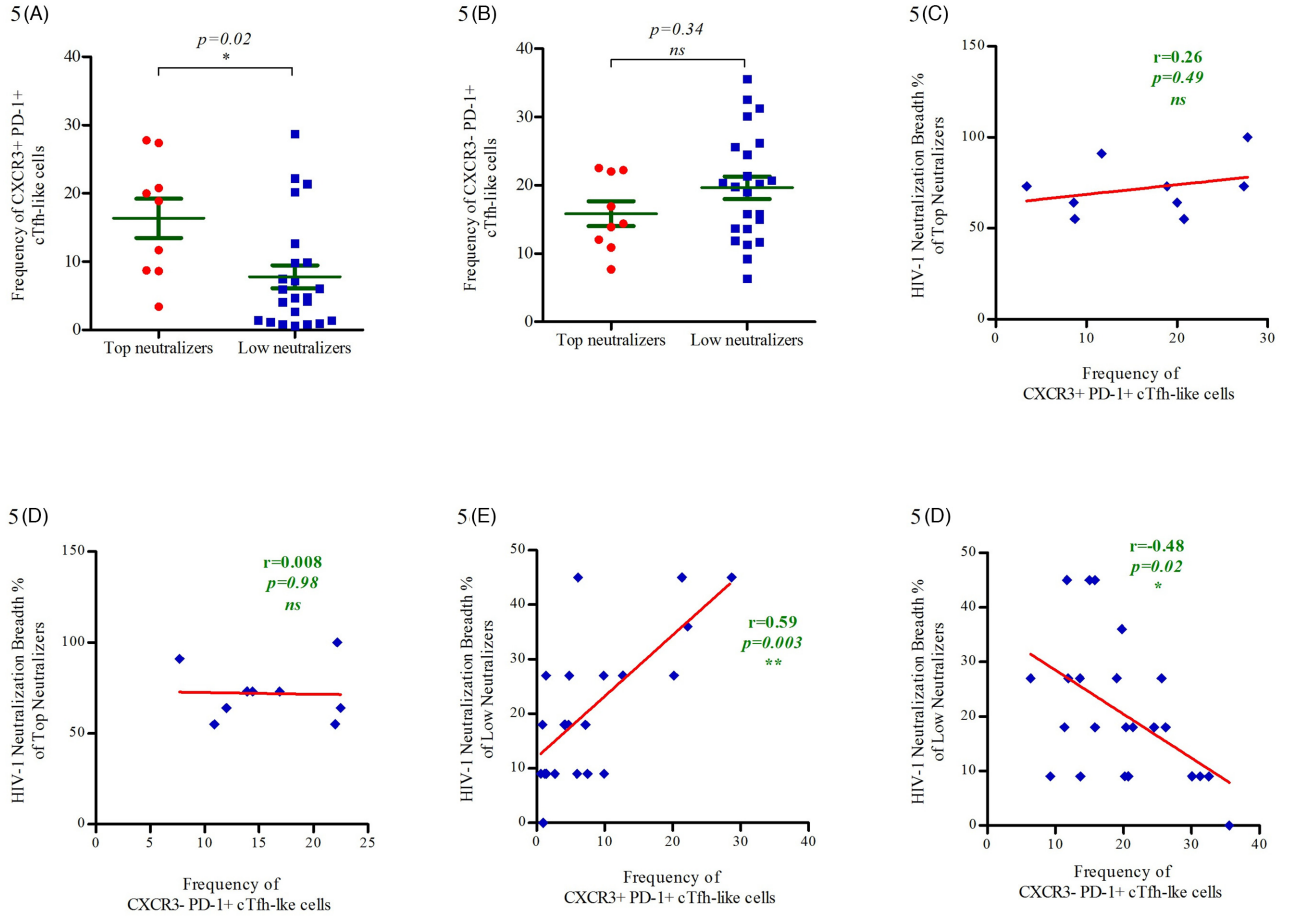

Figure 5. Based on the neutralization activity the cohort was subpopulated into Top and Low Neutralizers. Graphical plot showing the differences in frequencies of **(A)** CXCR3+PD-1+ cTfh-like cells and **(B)** CXCR3−PD-1+ cTfh-like cells between Top and Low Neutralizers. Correlations between cTfh-like cells and neutralization breadth were analyzed between **(C)** CXCR3+PD-1+ cTfh-like cells vs Neutralization Breadth of Top Neutralizers **(D)** CXCR3−PD-1+ cTfh-like cells vs Neutralization Breadth of Top Neutralizers **(E)** CXCR3+PD-1+ cTfh-like cells vs Neutralization Breadth of Low Neutralizers and **(F)** CXCR3−PD-1+ cTfh-like cells vs Neutralization Breadth of Low Neutralizers.

CXCR3 expressing CD4+ T-cells in blood were found to possess high levels of inducible replication competent virus even in aviremic ART treated HIV infected individuals. These CXCR3 + T-cells were also shown to express high levels of PD-1 directly correlating with HIV RNA from supernatants of viral outgrowth assay which suggests that PD-1 expression is driven by the presence of replication competent viruses in CXCR3 expressing CD4+ T-cells (Banga *et al.* 2018). Similarly, in this study presence of replication competent viruses in ART naïve progressors might have resulted in higher levels of CXCR3 expressing and PD-1expressing CD4+CXCR5+ T-cells. These results hypothesize that in LTNP controlled viral replication due to lower expression of CXCR3 and PD-1 in CD4+CXCR5+ T-cells could have been contributed for slower disease progression.

In this study, lower frequencies of CXCR3+PD-1+ cTfh-like cells were observed in LTNP, yet it strongly correlated with neutralization breadth and potency whereas the frequencies of CXCR3−PD-1+ cTfh-like cells were higher in LTNP but no correlations with bNAb breadth and potency were observed. It is reported that CXCR5+CXCR3+PD-1^{lo} cTfh-like cells expresses markers such as ICOS and Bcl-6 in lymphoid tissue and peripheral blood comparable to conventional CXCR5+CXCR3−PD-1^{hi} Tfh cells. In addition, though CXCR5+CXCR3+PD-1^{lo} Tfh-like cells were inferior to CXCR3− Tfh-like cells in Ig class switching, it can produce cytokines required for stimulating B-cell development and has the potential to differentiate into PD-1^{hi} Tfh-like cells. During conditions such as low viral loads and immune activation CXCR5+CXCR3+PD-1^{lo} cells may make important contributions to the induction and maintenance of bNAbs (Martin-Gayo *et al.* 2017). Similar phenomenon could have contributed to the association of CXCR3+PD-1+ cTfh-like cells with bNAbs in LTNP in this study. When categorized as top and low neutralizers, this study showed that in low neutralizers an increased neutralization breadth was observed when there is an increased CXCR3+PD-1+ cTfh-like cell proportion which gets decreased with increased CXCR3−PD-1+ cTfh-like cell frequencies. This reiterates the contribution of CXCR3+PD-1+ cTfh-like cells over CXCR3−PD-1+ cTfh-like cells towards neutralization activity in the cohort studied. A previous report indicated a transient increase in circulating ICOS+PD-1+CXCR3+ Tfh cells in blood correlating with protective antibody responses following influenza trivalent inactivated vaccinations (Bentebibel *et al.* 2013), thus supporting the hypothesis. Single positive CXCR3+ Tfh cells were shown to be enhanced in GC during chronic SIV infections. Increased CCR5 expressing CXCR3+ GC-Tfh cells were seen in chronic SIV infections indicating higher rate of SIV virion permissiveness of CXCR3 + cells (Velu *et al.* 2016). Similarly, CXCR3+CD4+ T cells preferentially expressed

the HIV-co-receptor CCR5 were also observed in the blood of HIV infected individuals as well as mouse model of HIV infection (Gosselin *et al.* 2010; Allam *et al.* 2015). Similarly, here persistent increase in low level viremia in LTNP, associated with increase in frequency of CXCR3+PD-1+ cTfh-like cells which in turn positively correlated with neutralization breadth and potency. Therefore, it might be suggested that, increasing frequency of CXCR3+PD-1+ cTfh-like cells in LTNP were able to maintain their functional properties of helping B-cells which in turn might induce bNAbs among disease nonprogressing LTNP. In progressors, there was absence of any association between CXCR3+PD-1+ cTfh-like cells and high-level viremia. These data suggest that an optimal induction of both CXCR3+PD-1+ and CXCR3−PD-1+ cTfh cells are required for sustained humoral responses through vaccinations. Ability of CXCR3+PD-1+ and CXCR3−PD-1+ cTfh-like cells to express IL-21 and their correlation with bNAb production would have provided more clarity on the role of these cTfh-like cells in this study.

CONCLUSION

The emergence of blood CXCR3+PD-1+ in CXCR5+CD4+ T cells correlates with the development of protective antibody responses in LTNP and showed no such correlation in progressors. These preliminary data suggest that CXCR3+PD-1+ cTfh-like cells might possess significant functional properties for driving B-cells to produce bNAbs in nonprogressing HIV infection. These observations may aid in knowing the outlining mechanisms required for inducing neutralizing antibodies by prophylactic vaccines.

LIMITATIONS

This study has a small sample size considering the practical difficulties in enrolling LTNP cohort. This is particularly a problem in our study, once we further stratified the LTNP group based on PVL. A longitudinal study with LTNP enrolled when they are in potential LTNP stage, might be effective in studying cTfh-like cells and its impact on neutralizing activity. Functional properties of cTfh-like cells were not studied due to lower amount of PBMC availability and limited resources.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSPD* online.

CONFERENCE PRESENTATIONS

The research abstract of the current paper was selected for poster presentation at the HIV Research for Prevention (HIVR4P 2018) conference held on 21st–25th October 2018 in Madrid, Spain (Abstract # A-0999-0001-00512).

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AUTHOR CONTRIBUTIONS

CRS and PN contributed in performing assays, literature search. CRS prepared the manuscript. RV contributed in experimental designing and data analysis. AKS contributed in recruiting the cohort. RG contributed in manuscript editing and review. HQ contributed in data analysis. SS performed clinical studies of the cohort and reviewed the manuscript. SSS contributed in data acquisition and provided facility support. LEH helped in using their facility for performing part of the assay. MPS provided technical support and helped in acquiring the flowcyometry data. NS, JM and SC contributed in manuscript review and editing, in addition to providing logistic and management support for study design and implementation. JK contributed in experimental design. KGM finalized the concept deign, reviewed the manuscript and is the corresponding author of the manuscript contributed in every aspect of the study.

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Conflicts of interest. None declared.

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