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# Follicular CD8+ T-cells are elevated in HIV infection and induce PD-L1 on B-cells

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## Abstract

Follicular $CD8^+CXCR5^+$ T-cells are a specialized $CD8^+$ T-cell subset with unique follicular-homing capabilities that have been reported to display effector functions in viral immunity, tumor immunity, and autoimmunity. $CD8^+CXCR5^+$ T-cells exhibit B-cell helper functions and express CD40L, ICOS, PD-1, and BCL-6, the transcriptional regulator of $CD4^+CXCR5^+$ T follicular helper ($T_{FH}$) cells and of germinal center B-cells. HIV is known to be sequestered in lymphoid follicles and $CD8^+CXCR5^+$ T-cell frequency is a marker for disease severity, given that HIV-infected patients with lower numbers of circulating $CD8^+CXCR5^+$ T-cells display lower $CD4^+$ T-cell counts. Likewise, several groups have reported a direct correlation between the quantity of $CD8^+CXCR5^+$ T-cells and suppression of HIV viral load. In this study, we observed elevated absolute numbers of $CD8^+CXCR5^+$ and $CD8^+CXCR5^+BCL\text{-}6^+PD\text{-}1^+$ T-cells in the blood of HIV-infected participants of the Multicenter AIDS Cohort Study (MACS). We further demonstrated *in vitro* that activated human $CD8^+CXCR5^+$ T-cells isolated from peripheral blood and tonsil from healthy donors show increased CD40L expression and induce the production of $PD\text{-}L1^+IgG^+$ B-cells. Moreover, absolute numbers of $CD8^+CXCR5^+$ T-cells significantly and positively correlated with numbers of $PD\text{-}L1^+$ B-cells found in blood of HIV-infected individuals. Altogether, these results show that activated $CD8^+CXCR5^+$ T-cells have the ability to activate

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Author contributions

L.E.M designed the study and performed co-culture experiments, flow cytometry, FACS analysis, data analysis, contributed to the interpretation of results, and manuscript writing; J.I preformed data analysis and manuscript writing; Y.G. performed experiments using the UCLA MACS repository samples; M.L.P contributed to the interpretation of the results and manuscript writing; and M.E. designed the study, performed data analysis, contributed to the interpretation of the results, and manuscript writing.

Disclosures

The remaining authors have declared that there are no disclosures or financial conflicts of interest with regard to this work.

B-cells and increase the percentage of PD-L1$^+$ and PD-L1$^+$IgG$^+$ B-cells, which provides insights into the early events of B-cell activation and differentiation, and may play a role in disease progression and lymphomagenesis in HIV-infected individuals.

## Introduction

CD8$^+$ T-cells are essential for the control of viral infections (1). Antigen specific CD8$^+$ T-cells differentiate into effector cytotoxic T-cells (CTLs), which can detect and kill virus-infected cells (1). However, in chronic HIV-infection, CTLs become dysfunctional and exhausted, and the accumulation of defective CD8$^+$ T-cells is thought to contribute to the systemic inflammation that is the hallmark of chronic HIV infection (2). A novel population of CD8$^+$ T-cells expressing CXCR5 present in secondary lymphoid tissues has been recently described (3–5). Functionally, CD8$^+$CXCR5$^+$ T-cells seem analogous to CD4$^+$ T follicular helper ($T_{FH}$) cells. $T_{FH}$ cells (CD4$^+$CXCR5$^+$PD-1$^+$BCL-6$^+$) are present in germinal centers in lymph nodes, where they interact with B-cells to refine antibody responses (6). $T_{FH}$ cells are part of the HIV reservoir: the pool of cells where HIV remains latent until reactivation (7). Follicular germinal centers, where $T_{FH}$ cells reside, were once thought to lack CD8$^+$ T-cells, thereby allowing HIV-infected $T_{FH}$ cells to escape CTL killing and to establish a HIV reservoir that evades immune system control (8). CD8$^+$ T-cells that express CXCR5, follicular CD8$^+$CXCR5$^+$ T-cells, have been shown to be present in secondary lymphoid tissues (4, 5) and interact with B-cells and promote antibody responses (9). CD8$^+$CXCR5$^+$ T-cells have been detected in lymphoid tissues of HIV-infected individuals, thus suggesting that they play a role in controlling HIV infection (4, 5, 10). The frequency of circulating HIV-specific CD8$^+$CXCR5$^+$ T-cells inversely correlates with HIV serum viral load, further suggesting a protective role (4, 5). Moreover, CD8$^+$CXCR5$^+$ T-cells display potent cytolytic activity to target and kill HIV-infected cells via bispecific antibodies *in vitro* (10).

During chronic viral infections, a sizable population of viral-specific CD8$^+$ T-cell clones acquire an exhausted profile characterized by reduced IL-2 production, impaired cytolytic function and proliferation, and increased expression of co-inhibitory receptors, such as the programmed cell death protein 1 (PD-1), Tim3, KLRG1, CD160, LAG3, and other markers of exhaustion (2, 11). Some evidence supports that CD8$^+$CXCR5$^+$ T-cells express high levels of PD-1 compared to CD8$^+$CXCR5$^-$ T-cells (4, 12, 13), while other reports suggest that CD8$^+$CXCR5$^-$ T-cells express slightly higher levels of PD-1 (5, 14). Interestingly, it has been shown that CD8$^+$CXCR5$^+$ T-cells maintain cytolytic capacity and are likely not exhausted (3, 15). Furthermore, blocking the PD-1/PD-L1 pathway during HIV infection diminished the function of CD8$^+$CXCR5$^+$ T-cells (12), which further suggests a protective role.

CD8$^+$CXCR5$^+$ T-cells play a number of roles during infection by promoting cytotoxic responses, supporting B-cell stimulating functions, and displaying CD8$^+$ T-effector memory characteristics (3). CD8$^+$CXCR5$^+$ T-cells present in human tonsils and follicular lymphoma samples reside in B-cell follicles, exhibit strong cytotoxic effects against tumor cells *in vitro*, can inhibit $T_{FH}$-mediated B-cell differentiation, and exert strong antitumor activity after adoptive transfer of CD8$^+$CXCR5$^+$ T-cells into an animal model of lymphoma compared

with its naïve and $CD8^+CXCR5^-$ counterparts (16). Recently, a colorectal cancer study revealed that $CD8^+CXCR5^+$ T-cells maintain cytolytic capacity to directly lyse tumor cells but also influence B-cell secretion of IgG, suggesting multiple mechanisms for tumor control by these cells (17). Others provide evidence of clinical correlations where high $CD8^+CXCR5^+$ T-cells in tumors or tumor draining lymph nodes correlate with increased colorectal cancer-free survival (18), disease-free pancreatic cancer survival after tumor resection (19), or high tumor expression of $CD8^+CXCR5^+$ T-cell signature genes that correlate with prolonged survival in follicular lymphoma (20).

Chronic B-cell activation associated with HIV infection is believed to contribute to the development of non-Hodgkin lymphoma (NHL) (21–23), and plays an important role in lymphomagenesis. Our prior work has shown that serum levels of inflammatory cytokines, such as IL-6, IL-10, IP-10/CXCL10, TNFα, sCD23, and CXCL13 (the ligand for CXCR5), are associated with increased risk for AIDS-associated NHL (AIDS-NHL) (24–28). We have also shown that regulatory B-cells (Bregs) are elevated prior to AIDS-NHL diagnosis, and that PD-L1-expressing B-cells comprise a subpopulation of Bregs (29). We are investigating chronic HIV infection and the early events leading to AIDS-NHL by elucidating the role of $CD8^+CXCR5^+$ T-cells on B-cell activation and differentiation and the early events of lymphomagenesis. In this study, we show that $CD8^+CXCR5^+$ T-cells are significantly elevated in peripheral blood of HIV-infected individuals part of the Multicenter AIDS Cohort Study (MACS). HIV-positive individuals also show elevated absolute numbers of $CD8^+CXCR5^+BCL\text{-}6^+PD\text{-}1^+$ T-cells compared to HIV-negative individuals. Moreover, we show that activated $CD8^+CXCR5^+$ T-cells can activate B-cells and promote the production of $PD\text{-}L1^+$ and $PD\text{-}L1^+IgG^+$ B-cells.

## Materials and Methods

### Multicenter AIDS Cohort Study (MACS) population

Viable frozen PBMCs were obtained from the UCLA Multicenter AIDS Cohort Study (MACS) repository. We obtained archival samples obtained from combination antiretroviral therapy (cART) naïve HIV+ participants ($n = 15$), as well as from HIV-negative controls ($n = 15$) whose race/ethnicity and age matched the HIV+ participants studied (Table I). Samples acquired from HIV+ participants were > 4 years prior to AIDS-NHL diagnosis.

### Flow cytometry of PBMCs isolated from MACS participants

Multicolor flow cytometry was performed on $10^6$ viable, thawed PBMCs collected from HIV+ and HIV-negative participants from the UCLA MACS repository. PBMCs were stained using a panel of extracellular immune cell markers, such as anti-CD3 eFluor 650 (clone OKT3; eBioscience), anti-CD4 PE-Cy7 (clone SK3; BD Biosciences), anti-CXCR5 (CD185) PerCP-Cyanine 5.5 (clone RF8B2; BD Pharmingen), anti-CD8 APC-eFluor 780 (or APC-Cy7) (clone RPA-T8; Invitrogen), anti-CD62L eFlour 605 (clone Dreg-56, Invitrogen), anti-PD-1 (CD279) APC (clone MIH4, eBioscience), anti-CD71 FITC (clone OKT9, eBioscience), anti-CD27 Pacific Blue (clone O323, Biolegend), and anti-ICOS (CD278) PE (clone ISA-3, eBioscience) for 20 minutes at 4°C, washed once in FACS buffer (0.5% bovine serum albumin (BSA) in PBS (Gibco), and then fixed with 1%

paraformaldehyde solution. A separate tube of cells was stained with the above antibody panel of extracellular immune cell markers that included anti-BCL-6 PE (clone K112–91, BD Pharmingen) for intracellular staining of BCL-6 (replacing anti-ICOS PE). For intracellular staining, the FoxP3 Transcription Factor Staining Buffer Set (eBioscience/Invitrogen) was used. Briefly, 1 ml of FoxP3 Fixation/Permeabilization working solution was added to each sample and incubated for 30–60 minutes at 2–8°C. After the incubation period, 2 ml of 1X Permeabilization buffer was added to each sample and centrifuged at 1,500 rpm for 5 minutes at room temperature, washed once more, and then stained with BCL-6 PE for 1 hour at room temperature. Cells were then washed once with FACS buffer and analyzed for flow cytometry using a BD LSRFortessa™ cell analyzer. Data presented in Figures 1, 2, and 5 were analyzed with the FCS Express software (v3, De Novo Software), and the gating strategy shown in Figure 1A was completed using the FCS Express software (v7.04.0004, De Novo Software). Absolute $CD3^+$ T-cell numbers were provided by the UCLA MACS, and absolute $CD4^+$ T-cell numbers for each HIV-negative or HIV+ participant was determined from the percentage of gated $CD3^+$ T-cells. Median $CD4^+$ T-cell numbers are provided for each group in Table I.

### Sorting $T_{FH}$ and $CD8^+CXCR5^+$ T-cells from HIV-negative PBMCs

PBMCs from healthy HIV-negative blood donors were obtained from the UCLA CFAR Virology Core. $10^8$ cells were used to sort $CD3^+CD4^+CD8^-CXCR5^+$ (follicular $CD4^+$ or $T_{FH}$) and $CD3^+CD4^-CD8^+CXCR5^+$ (follicular $CD8^+$) T-cells. PBMCs were stained with a panel of anti-human antibodies that included anti-CXCR5 Alexa Fluor 488 (clone RF8B2; BD Biosciences), anti-CD3 eVolve655 (clone OKT3; eBioscience), anti-CD4 PE-Cy7 (clone SK3; BD Biosciences), and anti-CD8 APC-780 (clone RPA-T8; eBioscience). After staining cells for 20 minutes at 4°C, cells were washed once with FACS buffer and resuspended in PBS. Cells were then sorted using a FACS Aria II cell sorter at the UCLA Jonsson Comprehensive Cancer Center (JCCC) Janis V. Giorgi Flow Cytometry Core Laboratory. For sorting of PBMCs and tonsil cells (below), the BD FACSDiva Software program (v8.02) was used. FlowJo (v10.8.1) was used to generate the gating strategy plots shown in Supplemental Figures 1 and 2.

### Co-culture of $T_{FH}$ or $CD8^+CXCR5^+$ T-cells with autologous primary B-cells

$CD19^+$ B-cells were isolated from PBMCs obtained from the same donor as used to obtain $T_{FH}$ or $CD8^+CXCR5^+$ T-cells. B-cells were isolated from a starting total of $5 \times 10^7$ PBMCs using an EasySep Human Pan B-cell Enrichment Kit according to the manufacturer's instructions (Stem Cell Technologies). Sorted $T_{FH}$ or $CD8^+CXCR5^+$ T-cells were plated at a density of $10^5$ cells per well in duplicate of a 96-well plate and $10^5$ $CD19^+$ B-cells were added to each well for a 1:1 T-cell to B-cell ratio in a final volume of 200 μl per well of complete RPMI-1640 medium (RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 Units/mL of penicillin (Gibco, Thermo Fisher Scientific), and 100 μg/mL of streptomycin (Gibco, Thermo Fisher Scientific)). Cells were cultured in the presence of IL-2 (20 Units/ml), with or without anti-CD3 and anti-CD28 coated dynabeads (Gibco) (1:1 bead-to-cell-ratio) for 2 days at 37°C, 5% $CO_2$. After 2 days of stimulation, cells were washed once with FACS buffer, and then resuspended in fresh FACS buffer. Cells were then stained for flow cytometry using the following anti-human antibody panel:

anti-CXCR5 Alexa Fluor 488 (clone RF8B2; BD Biosciences), anti-CD3 eVolve655 (clone OKT3; eBioscience), anti-CD4 PE-Cy7 (clone SK3; BD Biosciences), anti-CD8 APC-780 (clone RPA-T8; eBioscience), anti-CD19 PerCP-Cy5.5 (clone SJ25C1, eBioscience), and anti-PD-L1 APC (clone M1H1, eBioscience).

### Sorting and culture of tonsil $CD3^+CD4^-CD8^+CXCR5^-$ or $CD3^+CD4^-CD8^+CXCR5^+$ T-cells with $CD19^+IgM^+IgG^-$ B-cells

Tonsil specimens were obtained from healthy donors from the UCLA Ronald Reagan Medical Center (RRMC). Tonsils were processed by grinding the tissue and passing through a 70-μm cell strainer (Corning) with 1X PBS. The resulting cell suspension was mixed with 1X red blood cell (RBC) lysis buffer (Invitrogen) and incubated for 5 minutes at room temperature. Cells were then washed once in complete RPMI-1640 medium at 1,500 rpm for 5 minutes. Cells were counted and placed in two separate aliquots of $35 \times 10^6$ cells each in 5 ml polystyrene tubes, and then washed with FACS buffer and centrifuged at 1,500 rpm for 5 minutes. The two tubes consisting of $35 \times 10^6$ cells each were then stained with a panel of anti-human antibodies that included anti-CXCR5 Alexa Fluor 488 (clone RF8B2; BD Biosciences), anti-CD3 Super Bright 645 (clone OKT3; Invitrogen), anti-CD4 PE-Cy7 (clone SK3; BD Biosciences), anti-CD8 Alexa Fluor 700 (clone RPA-T8; eBioscience), anti-CD19 PerCP-Cyanine 5.5 (clone SJ25C1, eBioscience), anti-IgG PE (eBioscience), and IgM eFluor 450 (clone SA-DA4, eBioscience). After staining cells for 20 minutes at 4°C, cells were washed once with FACS buffer and resuspended in a final volume of 1.5 ml of 1X PBS. Cells were sorted using the FACS Aria II cell sorter to separate the following three cell populations: $CD3^+CD4^-CD8^+CXCR5^-$ T-cells, $CD3^+CD4^-CD8^+CXCR5^+$ T-cells, and $CD3^-CD19^+IgM^+IgG^-$ B-cells. The purity of these populations after sorting were as follows: $CD3^+CD4^-CD8^+CXCR5^-$ T-cells (93–95%); $CD3^+CD4^-CD8^+CXCR5^+$ T-cells (93–96%); and $CD3^-CD19^+IgM^+IgG^-$ B-cells (96–97%) across four independent experiments.

$CD3^+CD4^-CD8^+CXCR5^-$ or $CD3^+CD4^-CD8^+CXCR5^+$ T-cells were then co-cultured with $CD19^+IgM^+IgG^-$ B-cells at a 1:1 T-cell to B-cell ratio in complete RPMI-1640 medium and in the presence of IL-2 (20 Units/ml) plus anti-CD3 and anti-CD28 coated dynabeads (1:1 bead-to-cell-ratio) for 3 and 5 days (from the same sorted subsets) at 37°C, 5% $CO_2$. After 3 and 5 days, cells were harvested, split in thirds, and stained with three separate anti-human antibody panels for T- and B-cells. Panel 1: anti-CXCR5 Alexa Fluor 488 (clone RF8B2; BD Biosciences), anti-CD3 Super Bright 645 (clone OKT3; Invitrogen), anti-CD4 PE-Cy7 (clone SK3; BD Biosciences), anti-CD8 Alexa Fluor 700 (clone RPA-T8; eBioscience), anti-CD19 PerCP-Cy5.5 (clone SJ25C1, eBioscience), anti-PD-1 APC (clone EH12.2H7, BioLegend), and anti-CD40L PE (clone 89–76, BD Biosciences). Panel 2: the above antibody panel without anti-CD40L and with BCL-6 PE (clone K112–91, BD Biosciences) for intracellular staining. Panel 3: anti-CXCR5 Alexa Fluor 488 (clone RF8B2; BD Bioscience), anti-CD3 Super Bright 645 (clone OKT3; Invitrogen), anti-CD4 PE-Cy7 (clone SK3; BD Biosciences), anti-CD8 Alexa Fluor 700 (clone RPA-T8; eBioscience), anti-CD19 PerCP-Cy5.5 (clone SJ25C1, eBioscience), anti-PD-L1 APC (clone M1H1, eBioscience), anti-IgG PE (eBioscience), and anti-IgM eFlour 450 (Clone SA-DA4, eBioscience). Cells were stained for 20 minutes at 4°C, washed once in FACS buffer, and then resuspended in

2% PFA. For PBMC and tonsil cell experiments described above, cells were analyzed for flow cytometry using a BD LSRFortessa™ cell analyzer. Data was analyzed with the FCS Express software program (v7.04.0004, De Novo Software).

### Statistics

Graphical representations and statistical analysis were performed with GraphPad Prism 9.4.0. For data comparing HIV+ and HIV-negative study populations, unpaired, non-parametric Mann-Whitney tests (two-tailed) were performed (Fig. 1 and Fig. 2). For data acquired from PBMC experiments (B-cells co-cultured with $T_{FH}$ or $CD8^+CXCR5^+$ T-cells), an unpaired, non-parametric Kruskal-Wallis test was performed (Fig. 3). For data acquired from tonsil cell experiments of B-cells co-cultured with $CD8^+CXCR5^-$ or $CD8^+CXCR5^+$ T-cells, an unpaired, non-parametric Kruskal-Wallis test was conducted (Fig. 4). In one case (Fig. 4D), a statistical comparison was made using an unpaired, non-parametric Mann-Whitney test (two-tailed). For correlation analysis, two-tailed tests with 95% confidence interval were performed, where the Pearson correlation coefficient and *p*-value was determined (Fig. 5). *p*-values ≤ 0.05 were considered statistically significant in all cases.

## Results

### Characteristics of the MACS study population

HIV-positive participants and HIV-negative controls, who were participants in the UCLA Multicenter AIDS Cohort Study (MACS), were comparable in their demographic characteristics such as recruitment year, race, and ethnicity profiles (93% White, non-Hispanic and 7% Hispanic for HIV-negative controls vs. 90% White, non-Hispanic and 5% Hispanic for HIV+ individuals), and age group, which had medians of 34 and 38 years of age. All of the HIV+ individuals were combination antiretroviral therapy (cART) naïve (see Table I).

### $CD8^+CXCR5^+$ T-cells are elevated in peripheral blood of HIV+ participants and express BCL-6

We stained PBMCs from individuals living with untreated HIV infection to determine the absolute numbers of $CD8^+CXCR5^+$ T-cells and $CD8^+CXCR5^+PD\text{-}1^+$ T-cells at a single MACS visit ($n = 15$). $CD8^+CXCR5^+$, $CD8^+CXCR5^+PD\text{-}1^+$, and $CD8^+CXCR5^+PD\text{-}1^-$ T-cells were each gated from $CD3^+CD4^-CD8^+$ T-cells, as shown in Fig. 1A. Follicular $CD8^+$ T-cells accumulate in HIV-infected lymphoid follicles and germinal centers (4, 5, 9, 10 ), with minor fraction of these cells in HIV-negative individuals. In addition, others have shown that $CD8^+CXCR5^+$ T-cell quantity is elevated in peripheral blood of HIV-infected individuals compared with healthy controls (12). We observed that HIV+ participants had significantly elevated absolute numbers of $CD3^+CD4^-CD8^+CXCR5^+$ T-cells in blood when compared to HIV-negative individuals (Fig. 1B). The average percentage of $CD3^+CD4^-CD8^+$ that express $CXCR5^+$ T-cells in HIV-negative individuals (n = 15) was 3.21±1.45 (n = 15) and for those HIV+ was 2.42±1.71 (n = 15). The overall percentages were not significantly different. We found that the absolute numbers of $CD3^+CD4^-CD8^+CXCR5^+PD\text{-}1^+$ T-cells were also significantly elevated in HIV+

individuals compared to HIV-negative controls (Fig. 1C), while no significant differences in the absolute numbers of $CD3^+CD4^-CD8^+CXCR5^+PD\text{-}1^-$ T-cells was observed (Fig. 1D).

We then quantified the expression of BCL-6, a transcriptional factor that is characteristically expressed in $T_{FH}$ cells. We observed that $CD8^+CXCR5^+$ T-cells isolated from HIV+ and HIV-negative individuals expressed PD-1, CD27, and BCL-6 (Fig. 2A). HIV+ individuals had elevated absolute numbers of $CD8^+CXCR5^+BCL\text{-}6^+PD\text{-}1^+$ T-cells compared to HIV-negative individuals ($p = 0.021$) (Fig. 2B), and similar results were observed for the percentage of $CD8^+CXCR5^+BCL\text{-}6^+$ T-cells that express PD-1 (median percentage of 59.8% for HIV+ individuals vs. median percentage of 35.2% for HIV-negative individuals; $p = 0.015$).

## Activated $CD8^+CXCR5^+$ T-cells induce the production of $PD\text{-}L1^+$ and $PD\text{-}L1^+IgG^+$ B-cells and significantly correlate with absolute numbers of $PD\text{-}L1^+$ B-cells in HIV- infected individuals

Since $CD8^+CXCR5^+$ T-cells share phenotypic characteristics with $T_{FH}$ cells, it is possible that they may also share functional features. To test this hypothesis, we determined whether $CD8^+CXCR5^+$ T-cells can induce B-cells to express PD-L1, as $T_{FH}$ cells do. $PD\text{-}L1^+$ B-cells are known to be elevated during HIV infection (29). We isolated PBMCs from healthy donors and sorted $CD3^+CD4^+CD8^-CXCR5^+$ ($T_{FH}$ cells) (positive control) and $CD3^+CD4^-CD8^+CXCR5^+$ ($CD8^+CXCR5^+$) T-cells from the same donor (Supplemental Fig. 1). $T_{FH}$ or $CD8^+CXCR5^+$ T-cells were co-cultured with naïve $CD3^-CD19^+$ B-cells isolated from the same donor (1:1 T-cell to B-cell ratio) in the presence of IL-2 and in the presence or absence of anti-CD3/anti-CD28 coated dynabeads. As shown in Fig. 3A, $T_{FH}$ cells induced expression of PD-L1 on B-cells and this induction was more pronounced when $T_{FH}$ cells were activated with both IL-2 and anti-CD3/anti-CD28 (Fig. 3A), as has been shown by others (30, 31). Activated $CD8^+CXCR5^+$ T-cells were also capable of significantly inducing PD-L1 expression on B-cells, and this induction was also more pronounced when $CD8^+CXCR5^+$ T-cells were activated with both IL-2 and anti-CD3/anti-CD28 (Fig. 3B).

To further investigate whether $CD8^+CXCR5^+$ T-cells from secondary lymphoid organs are capable of inducing PD-L1 expression on B-cells, tonsil cells were stained and sorted for $CD3^+CD4^-CD8^+CXCR5^-$ and $CD3^-CD4^-CD8^+CXCR5^+$ T-cells, and $CD3^-CD19^+IgM^+IgG^-$ B-cell populations (Supplemental Fig. 2). It has been reported that $CXCR5^+PD\text{-}1^+CD8^+$ T-cells provide help to B-cells for the production of immunoglobulins, such as IgG1 (32). Thus, $CD8^+CXCR5^-$ or $CD8^+CXCR5^+$ T-cells were co-cultured with $CD19^+IgM^+IgG^-$ B-cells at a 1:1 ratio in the presence of IL-2 plus anti-CD3/anti-CD28 dynabeads for 3 and 5 days. We found that activated $CD8^+CXCR5^+$ T-cells significantly induced the percentage of $CD19^+PD\text{-}L1^+$ B-cells at 3 (Fig. 4A) and 5 days of co-culture (Supplemental Fig. 3A). Moreover, we determined a significantly higher percentage of $CD19^+PD\text{-}L1^+IgG^+$ B-cells after culture with activated $CD8^+CXCR5^+$ T-cells compared to co-cultures with activated $CD8^+CXCR5^-$ T-cells or B-cells alone and in the absence of activated $CD8^+$ T-cells at 3 (Fig. 4B) and 5 days (Supplemental Fig. 3B).

The ability of $CXCR5^+PD\text{-}1^+CD8^+$ T-cells to help B-cells depends on the CD40/CD40L interaction (32). Thus, we further measured the expression of CD40L on $CD8^+CXCR5^-$ and

$CD8^+CXCR5^+$ T-cells after co-culture with B-cells. We found that in most cases, a higher percentage of $CD40L^+$ cells were observed among activated $CD8^+CXCR5^+$ T-cells after 3 (Fig. 4C, 4D) and 5 days of co-culture (Supplemental Figure 3).

Considering that $CD8^+CXCR5^+$ T-cells can induce PD-L1 expression on B-cells and $PD\text{-}L1^+$ B-cells are also elevated during HIV infection (29), we further investigated whether $CD8^+CXCR5^+$ T-cells correlated with the numbers of PD-L1-expressing B-cells in peripheral blood of HIV-infected individuals. We found that the absolute numbers of $CD8^+CXCR5^+$ T-cells significantly and positively correlated with the absolute numbers of $PD\text{-}L1^+$ B-cells (Fig. 5).

## Discussion

In this study, we describe the presence of $CD8^+CXCR5^+$ T-cells in peripheral blood during HIV infection. Utilizing flow cytometry, we analyzed these cells in the peripheral blood of 15 HIV-infected participants, who were naïve to cART, as well as in a group of 15 HIV-negative controls. Both groups were matched for race/ethnicity and age (Table I).

We observed that the numbers of $CD8^+CXCR5^+$ T-cells were elevated in the peripheral blood of HIV-infected individuals. $CD8^+CXCR5^+$ T-cells have been previously shown to be elevated in lymphoid tissue of HIV-infected individuals (4, 5). During HIV infection, $CD8^+CXCR5^+$ T-cells in lymph nodes have been described to be HIV-specific and to play an important role in HIV clearance (4, 5). The higher number of $CD8^+CXCR5^+$ T-cells in the blood of HIV-infected individuals is of importance, as this suggests that these cells migrate from the lymphoid tissue during HIV infection. Although the reason for the presence of these cells in the peripheral circulation is unknown, it is known that CXCL13, the natural ligand of CXCR5, is elevated in the serum of HIV-infected individuals (33). Therefore, it is possible that the elevated levels of CXCL13 seen in blood of HIV-infected individuals may be leading $CD8^+CXCR5^+$ T-cells to traffic out of the lymphoid tissue into the peripheral circulation. Additionally, the architecture of secondary lymphoid tissues is disrupted in progressive untreated HIV infection (34), which may result in the relocation to peripheral circulation of cells that would normally reside in these tissues. We also observed a higher percentage of $CD8^+CXCR5^+PD\text{-}1^+$ T-cells in HIV-infected individuals.

The role of $CD8^+CXCR5^+$ T-cells in the control of HIV infection can be explained, at least in part, by the fact that these cells express BCL-6. BCL-6 is the transcriptional factor characteristic of $T_{FH}$ cells (6) and $CD8^+CXCR5^+$ T-cells residing in the lymph nodes have been ascribed to provide help promoting B-cell activation and antibody secretion (9, 35). HIV infection increases the number of $CD8^+CXCR5^+$ T-cells and these cells presumably have an active role in the maturation of specific anti-HIV B-cells. During chronic HIV infection, $CD8^+CXCR5^+$ T-cells can become exhausted, as indicated by the expression of PD-1 and other markers of exhaustion, thus, adding to the pool of differentiated, dysfunctional $CD8^+$ T-cells that are characteristically seen in HIV infection.

$CD8^+CXCR5^+$ T-cells are phenotypically similar to $T_{FH}$ cells. Therefore, we wanted to determine whether $CD8^+CXCR5^+$ T-cells can also exert a similar function. The main

function of $T_{FH}$ cells is to help B-cells in the germinal center, where they induce B-cells to undergo class switch recombination and somatic hypermutation, which eventually induce B-cells to secrete specific antibodies (6). The interaction between $T_{FH}$ and B-cells is tightly regulated. To study whether $CD8^+CXCR5^+$ T-cells share functions with $T_{FH}$ cells, we investigated whether $CD8^+CXCR5^+$ T-cells could induce PD-L1 expression on B-cells as $T_{FH}$ cells are capable of doing so. We found that $CD8^+CXCR5^+$ T-cells isolated from peripheral blood and a secondary lymphoid organ, such as the tonsil, can induce the generation of PD-$L1^+IgG^+$ B-cells. Our data also show that activated $CD8^+CXCR5^+$ T-cells express CD40L, further suggesting a possible mechanism for activating B-cells. Moreover, we determined that the number of $CD8^+CXCR5^+$ T-cells correlated with the number of PD-$L1^+$ B-cells isolated from HIV-infected individuals. As PD-$L1^+$ B-cells are elevated in HIV infection, and more so in individuals who are HIV+ and go onto develop lymphoma (29), the activation of B-cells by $CD8^+CXCR5^+$ T-cells may contribute to immune dysfunction and pathogenesis in HIV infection. PD-L1-expressing B-cells may exert regulatory functions by interacting with PD-1 and limiting T-cell activation.

In summary, $CD8^+CXCR5^+$ T-cells, a novel population of B-helper cells, merit further study as the function of these cells in those living with chronic, untreated HIV infection may contribute to progressive HIV disease. Therefore, more precisely determining the function of these cells may provide important insights on the immune control of HIV infection. Additionally, these cells may be important contributors to the general immune activation present during the course of HIV infection, which contributes to the many co-morbidities seen in HIV-infected individuals.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Key points**

- $CD8^+CXCR5^+$ T-cells are elevated in HIV-positive MACS participants.
- Activated $CD8^+CXCR5^+$ T-cells induce PD-$L1^+$ and PD-$L1^+IgG^+$ B-cells.
- $CD8^+CXCR5^+$ T-cells significantly and positively correlate with PD-$L1^+$ B-cells.

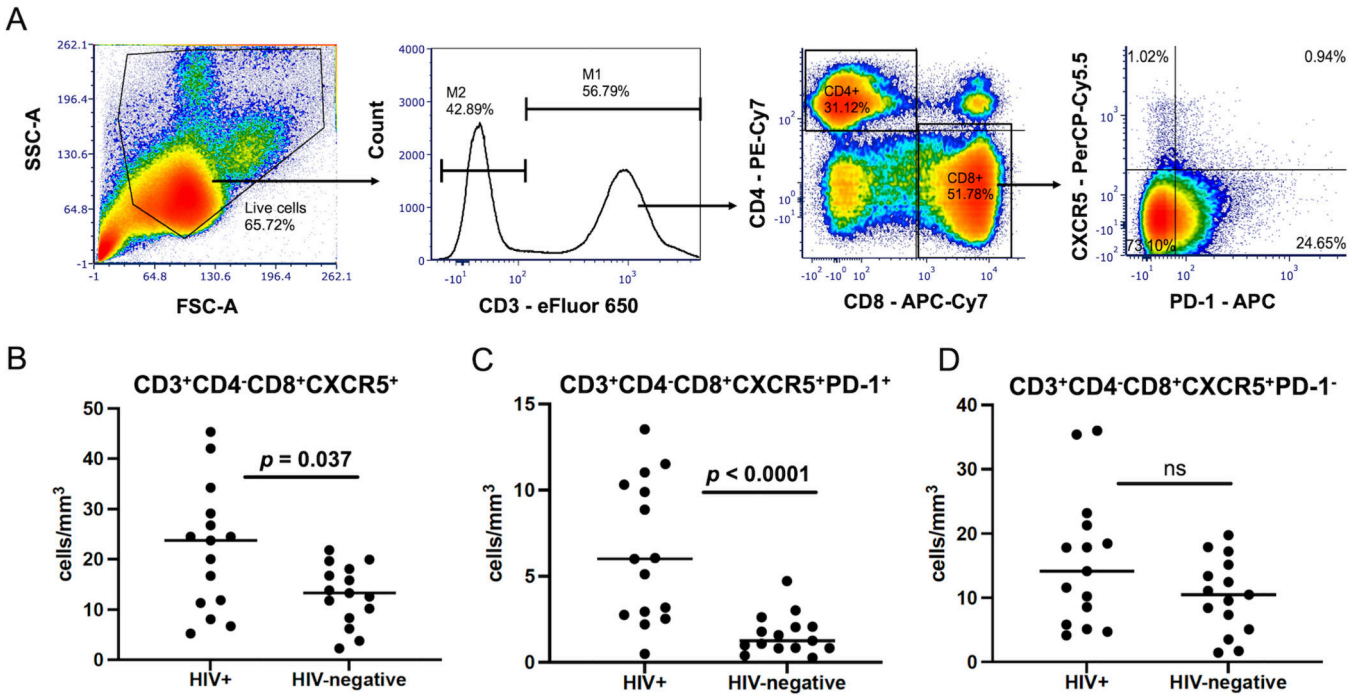


**Figure 1. $CD3^+CD4^-CD8^+CXCR5^+$ T-cells are elevated in peripheral blood of HIV-infected individuals part of the UCLA MACS.**

Multi-color flow cytometry was performed on viable, thawed PBMCs from HIV+ participants ($n = 15$) and HIV-negative controls ($n = 15$) from the UCLA MACS. (**A**) Shown is the representative gating strategy used to gate $CD3^+$ T-cells from live cells, $CD4^+$ and $CD8^+$ T-cells from gated $CD3^+$ T-cells, and $CD8^+CXCR5^+$ and $CD8^+CXCR5^+PD\text{-}1^{+/-}$ T-cells, which were gated from $CD3^+CD4^-CD8^+$ T-cells. Absolute numbers of $CD3^+CD4^-CD8^+CXCR5^+$ T-cells (cells/$mm^3$) (**B**), $CD3^+CD4^-CD8^+CXCR5^+PD\text{-}1^+$ (**C**), and $CD3^+CD4^-CD8^+CXCR5^+PD\text{-}1^-$ (**D**) T-cells were measured in HIV+ ($n = 15$) and HIV-negative ($n = 15$) individuals. Absolute numbers were determined from the percentage of gated $CD3^+CD4^-CD8^+$ T-cells in HIV+ and HIV-negative controls. Lines represent median values of the given measurement for each group. *p*-values were determined using unpaired, non-parametric Mann-Whitney test (two-tailed).

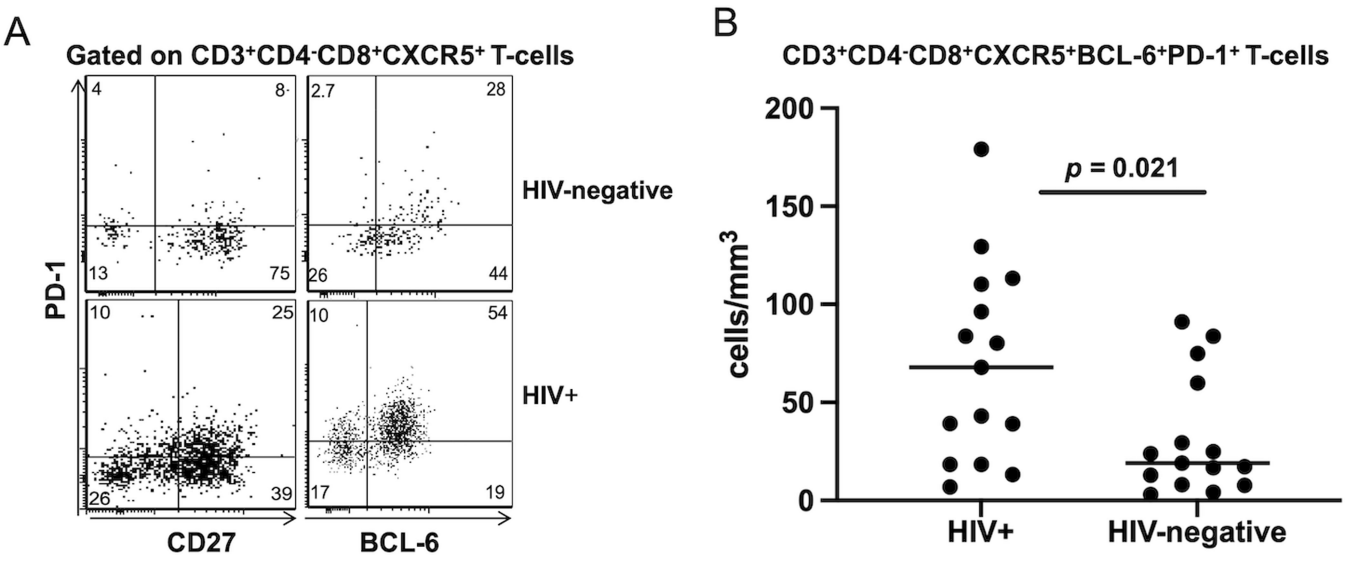


**Figure 2. CD3$^+$CD4$^-$CD8$^+$CXCR5$^+$ T-cells isolated from HIV-positive individuals express BCL-6.**

Multi-color flow cytometry was performed using viable, thawed PBMCs isolated from HIV-negative and HIV+ participants from the UCLA MACS. (**A**) CD3$^+$CD4$^-$CD8$^+$CXCR5$^+$ T-cells were further sub-gated to identify PD-1$^+$, PD-1$^+$CD27$^+$, and PD-1$^+$BCL-6-expressing cell populations. Flow cytometry plots are representative of HIV-negative ($n$ = 15) and HIV-positive individuals ($n$ = 15) that were studied. (**B**) Absolute numbers of CD3$^+$CD4$^-$CD8$^+$CXCR5$^+$BCL-6$^+$PD-1$^+$ (cells/mm$^3$) are provided for HIV-negative and HIV-positive individuals ($p$ = 0.021). Lines represent median values for each group. The $p$-value was determined using an unpaired, non-parametric Mann-Whitney test (two-tailed).

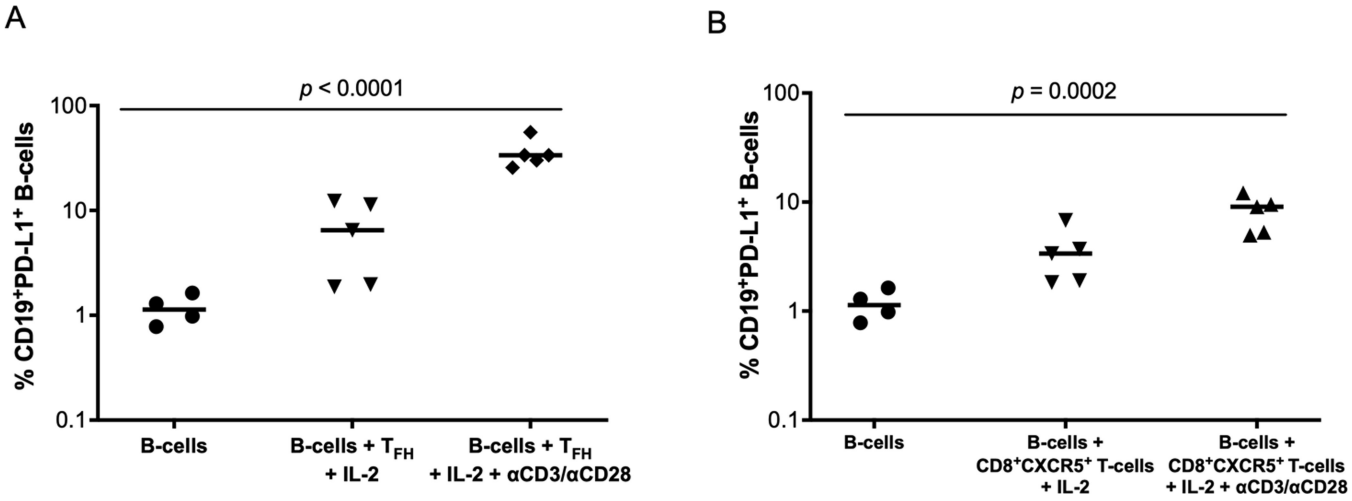


**Figure 3. Activated $T_{FH}$ and follicular $CD8^+$ T-cells significantly induce the percentage of $CD19^+PD\text{-}L1^+$ B-cells.**

(**A-B**) Sorted $CD3^+CD4^+CD8^-CXCR5^+$ ($T_{FH}$) and $CD3^+CD4^-CD8^+CXCR5^+$ (follicular $CD8^+$) T-cells were co-cultured with $CD3^-CD19^+$ B-cells isolated from the same healthy blood donor at a ratio of 1:1 in the presence of IL-2 (20 U/ml) and in the presence or absence of anti-CD3/anti-CD28 (αCD3/αCD28) dynabeads (1:1 bead-to-cell ratio) for 2 days. Dot plots show the percentage of $CD19^+PD\text{-}L1^+$ B-cells measured after 2 days of co-culture with $T_{FH}$ (**A**) or $CD8^+CXCR5^+$ T-cells (**B**). Lines represent median values of the given measurement for each group. Results are from four (B-cells only) or five independent experiments using different healthy blood donors (PBMCs). Experimental duplicates were done for each experiment and pooled to have sufficient cells to stain for flow cytometry. Thus, data is shown as an individual dot for each experiment. *p*-values are provided and were determined using an unpaired, non-parametric Kruskal-Wallis test. The median values varied significantly across the three groups ($p < 0.05$).

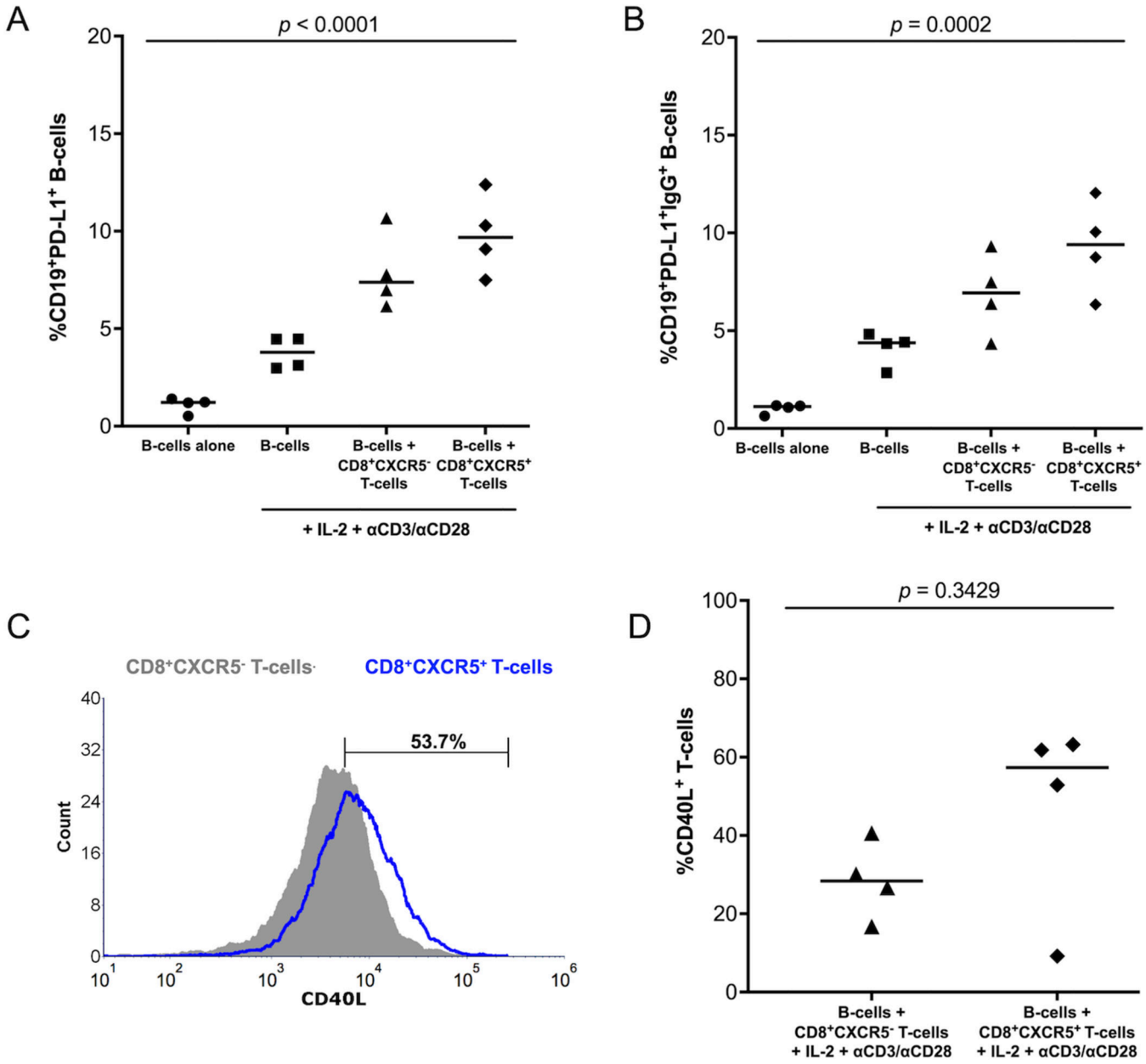


**Figure 4. Activated tonsil CD8$^+$CXCR5$^+$ T-cells significantly induce the percentage of PD-L1$^+$ and PD-L1$^+$IgG$^+$ B-cells.**

(**A-D**) Sorted tonsil CD3$^+$CD4$^-$CD8$^+$CXCR5$^-$ or CD3$^+$CD4$^-$CD8$^+$CXCR5$^+$ T-cells were co-cultured with sorted tonsil CD19$^+$IgM$^+$IgG$^-$ B-cells at a ratio of 1:1 in the presence of IL-2 (20 U/ml) plus anti-CD3/anti-CD28 (αCD3/αCD28) dynabeads (1:1 bead-to-cell ratio) for 3 days. Paired dot plots show the percentage of CD19$^+$PD-L1$^+$ B-cells (**A**) and the percentage of CD19$^+$PD-L1$^+$IgG$^+$ B-cells (**B**) at 3 days of co-culture. Representative histogram of CD40L (**C**) and dot plots showing the percentage of CD40L$^+$ among CD8$^+$CXCR5$^-$ and CD8$^+$CXCR5$^+$ T-cells that were co-cultured with CD19$^+$IgM$^+$IgG$^-$ B-cells for 3 days (**D**), where $p = 0.3429$ (not significant). Data are from four independent experiments conducted from four different tonsil specimens. Experimental duplicates were

done for each experiment and pooled to have sufficient cells to stain for flow cytometry. Thus, data is shown as an individual dot for each experiment. Statistical comparisons for **A** and **B** were made using an unpaired, non-parametric Kruskal-Wallis test. Statistical comparisons for **D** were made using an unpaired, non-parametric Mann-Whitney test (two-tailed).

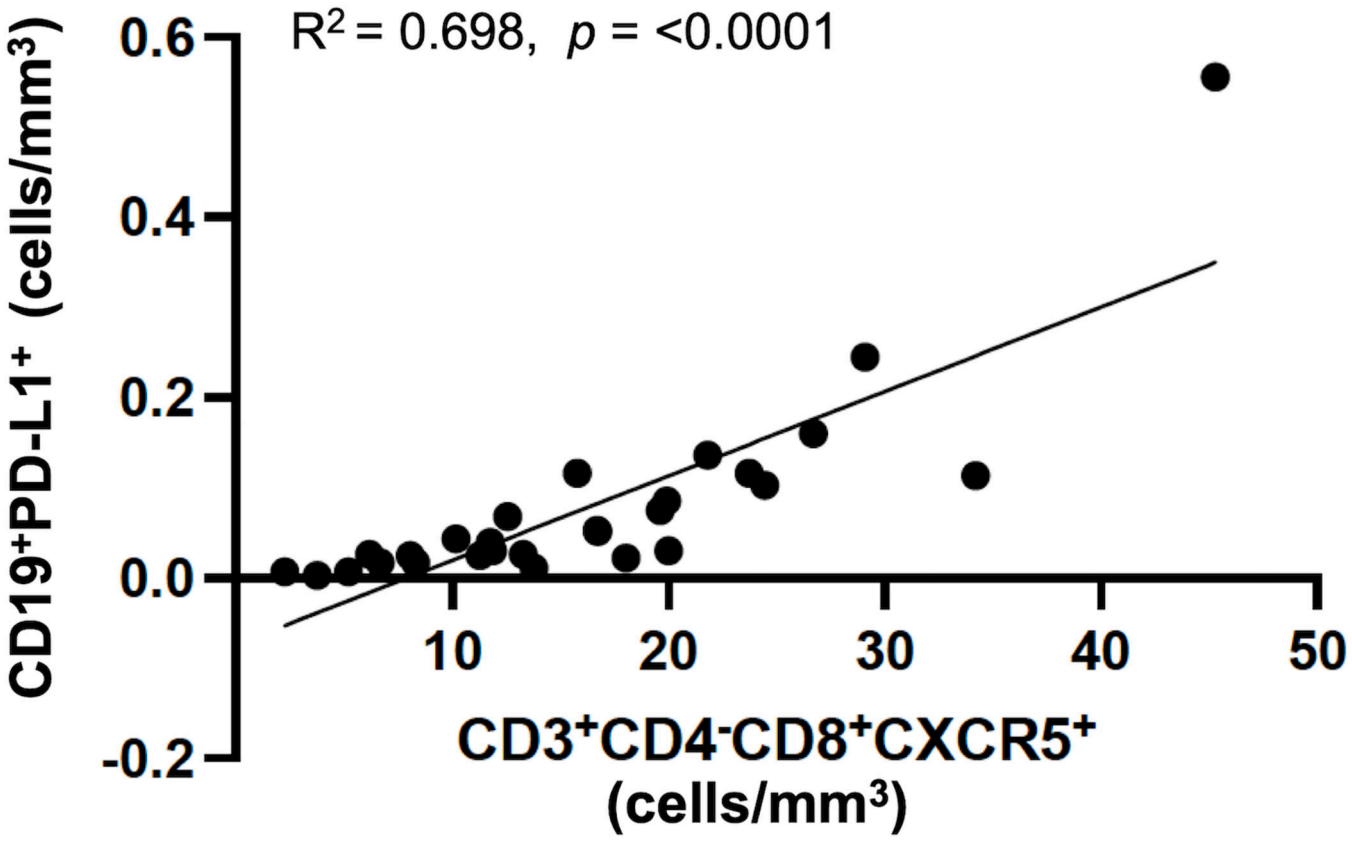


**Figure 5. $CD3^+CD4^-CD8^+CXCR5^+$ T-cells significantly and positively correlate with $CD19^+PD\text{-}L1^+$ B-cells.**

Multi-color flow cytometry was performed on viable frozen PBMCs acquired from HIV+ individuals ($n = 15$) and HIV-negative controls ($n = 15$) from the UCLA MACS. The linear regression plot shows absolute numbers of $CD3^+CD4^-CD8^+CXCR5^+$ T-cells (cells/$mm^3$) with absolute numbers of $CD19^+PD\text{-}L1^+$ B-cells (cells/$mm^3$). A two-tailed correlation analysis with 95% confidence interval was performed, where the Pearson correlation coefficient was $R^2 = 0.698$ and $p < 0.0001$.

**Table 1.**

Select characteristics of the MACS study population.

| | HIV-negative controls | HIV+ individuals |
|---|---|---|
| | ***n* = 15** | ***n* = 15** |
| **Age (years) median ± SD** [a] | 38 ± 9 | 39 ± 6 |
| **Sex** | | |
| male | 100% | 100% |
| female | 0% | 0% |
| **CD4⁺T-cell count (cells/mm³), median ± SD** [*] | 975 ± 169 | 333 ± 242 |
| **cART** [b] | | |
| Yes | n/a[c] | 0% |
| No | n/a | 100% |
| **Race and ethnicity** | | |
| White, non-Hispanic | 93% | 90% |
| Black, non-Hispanic | 0% | 0% |
| Hispanic | 7% | 5% |
| Other | 0% | 5% |

[*] Values for each participant at the PBMC sample date > 4 years prior to AIDS-NHL diagnosis

[a.] Median ± SD: Median± standard deviation

[b.] cART: Combination antiretroviral therapy

[c.] n/a: not applicable