

# Circulating CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells in HIV-1 controllers with neutralizing antibody breadth

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**HIV-1-specific broadly neutralizing antibodies (bnAbs) typically develop in individuals with continuous high-level viral replication and increased immune activation, conditions that cannot be reproduced during prophylactic immunization. Understanding mechanisms supporting bnAb development in the absence of high-level viremia may be important for designing bnAb-inducing immunogens. Here, we show that the breadth of neutralizing antibody responses in HIV-1 controllers was associated with a relative enrichment of circulating CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> CD4<sup>+</sup> T cells. These CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells were preferentially induced in vitro by functionally superior dendritic cells from controller neutralizers, and able to secrete IL-21 and support B cells. In addition, these CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells contained higher proportions of stem cell-like memory T cells, and upon antigenic stimulation differentiated into PD-1<sup>hi</sup> Tfh-like cells in a Notch-dependent manner. Together, these data suggest that CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> cells represent a dendritic cell-primed precursor cell population for PD-1<sup>hi</sup> Tfh-like cells that may contribute to the generation of bnAbs in the absence of high-level viremia.**

## Introduction

Antibodies with broad neutralizing activity against different strains of HIV-1 (bnAbs) (1, 2) represent immune responses that, in principle, could be reproduced in healthy individuals to prevent infection with HIV-1. However, mechanisms required to generate and maintain such bnAbs seem extremely complex, and remain poorly understood. Follicular CD4<sup>+</sup> T helper (Tfh) cells are critical for priming of B cell responses within lymph node germinal centers, which leads to the development of bnAbs (3, 4). Tfh cells are phenotypically characterized by the expression of the surface receptor CXCR5, and their developmental program is regulated by the master transcription factor Bcl-6 (5, 6). Functionally, Tfh cells enhance maturation, Ig class switching, and affinity maturation in B cells by secreting cytokines such as IL-21 and IL-4 (7, 8), and through contact-dependent mechanisms (9, 10). The molecular and cellular signals necessary for Tfh development represent an area of active investigation, but current data from experimental animal models suggest that antigen presentation by DCs is necessary and sufficient to initiate a Tfh development program (11, 12), while cognate interactions with activated B cells seem required to sustain DC-primed Tfh cells (13).

Tfh cells reside in lymphoid tissue (14), but a population of CXCR5<sup>+</sup>PD-1<sup>+</sup>CD4<sup>+</sup> T lymphocytes circulating in the peripheral blood has been proposed to act as peripheral counterparts of Tfh cells (pTfh cells) (15, 16). In comparison to germinal center Tfh cells, peripheral blood CXCR5<sup>+</sup>CD4<sup>+</sup> T

**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Submitted:** July 15, 2016

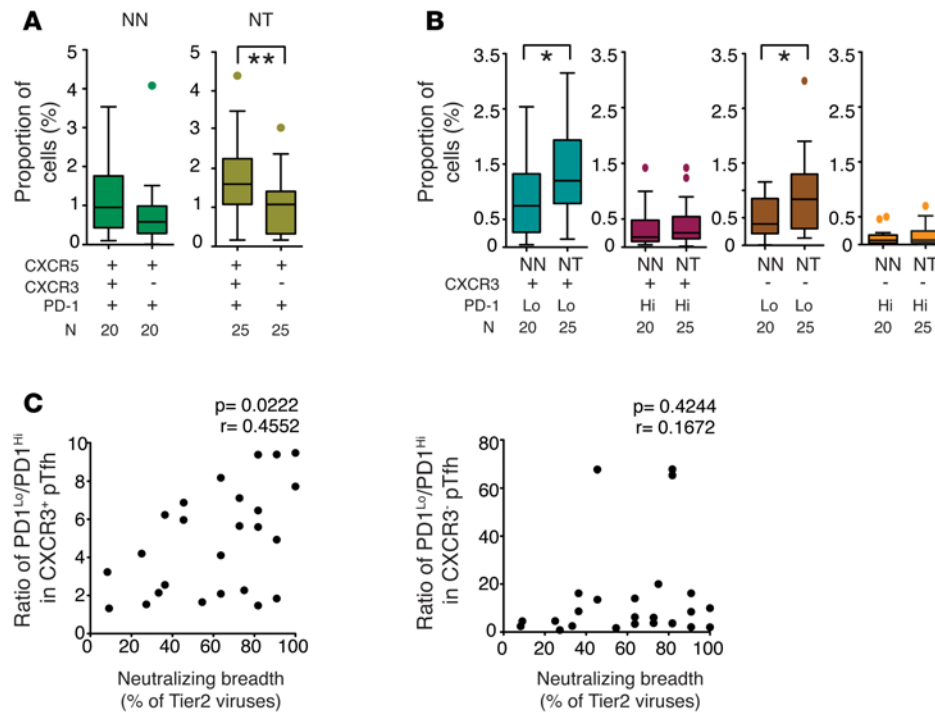
**Accepted:** December 12, 2016

**Published:** January 26, 2017

**Reference information:**

*JCI Insight.* 2017;2(2):e89574.

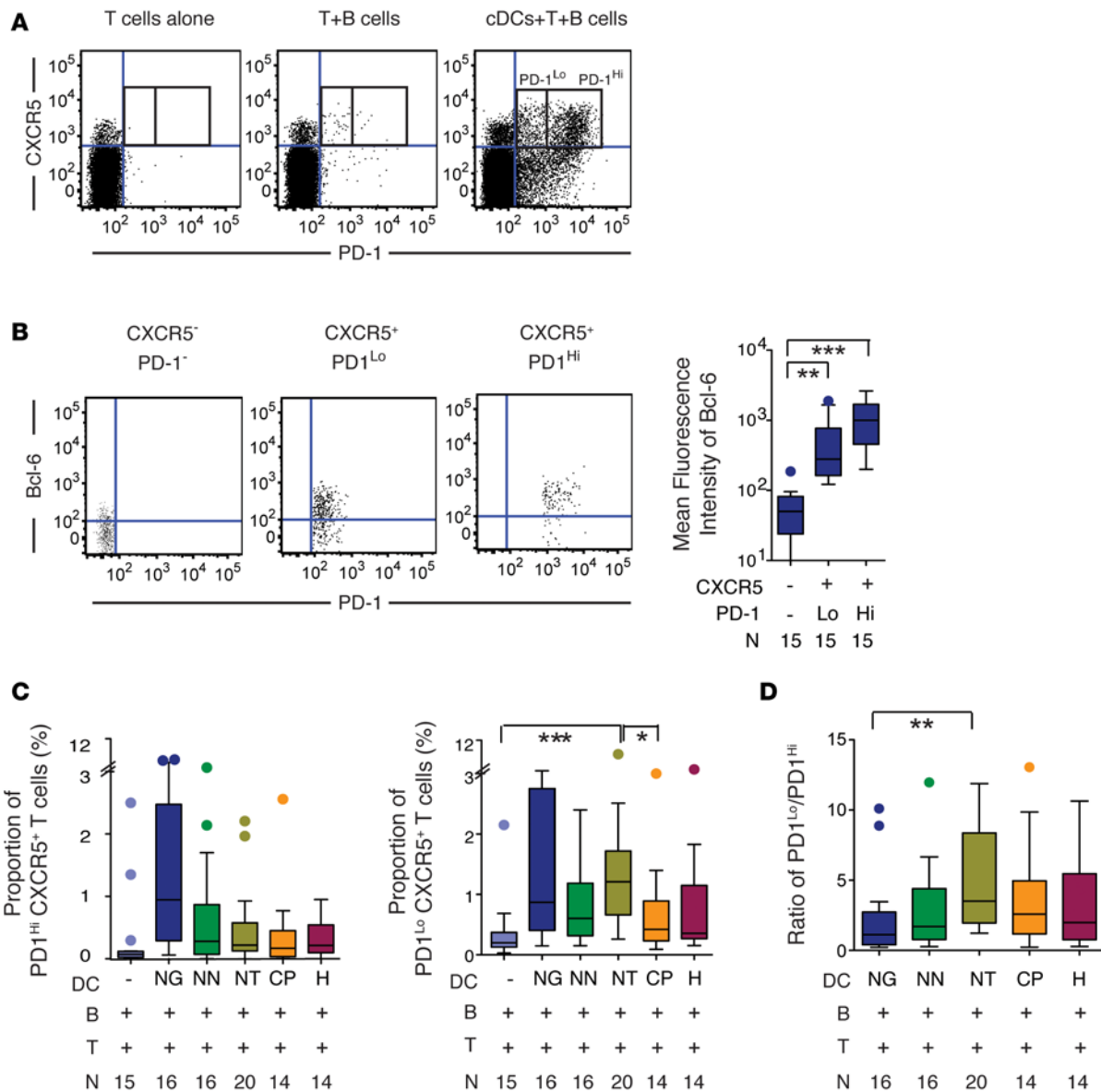
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**Figure 1. CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells are enriched in controller neutralizers and associated with neutralizing breadth against HIV-1.** (A) Proportions of total CXCR3<sup>+</sup>PD-1<sup>+</sup>CXCR5<sup>+</sup> or CXCR3<sup>+</sup>PD-1<sup>+</sup>CXCR5<sup>+</sup> subpopulations within total CD4<sup>+</sup> T cells in blood from HIV-1-infected controller non-neutralizers (NN, *n* = 20) and neutralizers (NT, *n* = 25). Statistical differences were calculated using a Wilcoxon matched-pairs test in each group of patients (\*\**P* < 0.01). (B) Proportions of PD-1<sup>lo</sup> or PD-1<sup>hi</sup> cells within CXCR3<sup>+</sup>CXCR5<sup>+</sup> and CXCR3<sup>-</sup>CXCR5<sup>+</sup> CD4<sup>+</sup> T cell populations from the indicated study cohorts. Statistical differences between NN and NT were calculated separately for each individual pTfh subset using a Mann Whitney *U* test (\**P* < 0.05). (C) Spearman correlations between ratios of PD-1<sup>lo</sup> vs PD-1<sup>hi</sup> cells within CXCR3<sup>+</sup> (left) and CXCR3<sup>-</sup> (right) CXCR5<sup>+</sup> CD4<sup>+</sup> T cells from controller neutralizers and the corresponding breadth of HIV-1-specific neutralizing antibodies. Numbers included in the top right corner of each plot reflect nominal *P* values and Spearman *r* values.

cells express reduced levels of ICOS, Bcl-6, and cellular activation markers such as CD69 and HLA-DR, but maintain the ability to stimulate Ab production and Ig class switching in B cells in vitro upon reactivation with cognate antigens (15, 17), suggesting that they represent Tfh-committed memory cells. pTfh cells have been further subdivided into distinct subsets based on expression of CXCR3 and CCR6 receptors, but the contribution of each subtype to the development of humoral immunity remains controversial (16–19). In HIV-1 infection, associations between circulating CXCR5<sup>+</sup>CXCR3<sup>-</sup>PD-1<sup>+</sup> Tfh cells and the breadth of HIV-1-specific neutralizing antibodies were made in a cohort of chronically infected individuals with continuously ongoing high plasma viral loads and high immune activation (16). In contrast, following immunization with influenza vaccines (19) or HPV vaccines (20) (i.e., during more limited antigen exposure), humoral immune responses were correlated with CXCR3<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> CD4<sup>+</sup> T cells, and CXCR3<sup>+</sup>CXCR5<sup>+</sup> CD4<sup>+</sup> T cells were also observed in blood and lymph nodes in rhesus macaques immunized with an SIV vaccine (21). In addition, recent studies in non-human primate models also reported induction of CXCR3<sup>+</sup> Tfh in chronic SIV infection (22). Therefore, the contribution of pTfh subsets to the development of protective Ab responses seems to be context dependent and requires further investigation.

HIV-1 controllers are able to spontaneously maintain low or undetectable levels of viral replication and arguably provide the most informative opportunity to study effective HIV-1 immune defense mechanisms. Most prior studies in these patients have focused on cellular mechanisms of antiviral immune control and identified highly functional HIV-1-specific memory CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses as the predominant correlate of antiviral immune defense (23); this represents a sharp contrast to HIV-1 progressors, in whom there is considerable evidence for a defective and functionally exhausted memory cell response to HIV-1. Mechanisms of HIV-1-specific humoral immunity and memory pTfh cells in HIV-1 controllers remain largely uncertain, although prior studies noted that the development of HIV-1-specific antibodies with increased neutralizing breadth seems rare in these patients (24). In the present study, we show that relative enrichment of CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> CD4<sup>+</sup> T cells is associated with increased HIV-1 neutralizing antibody breadth in controllers. Importantly, CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells were efficiently primed by myeloid DCs (mDCs) from HIV-1 controller neutralizers, were phenotypically enriched for immature, stem cell-like CD4<sup>+</sup> T cells, and were able to partially support B cell differentiation and secreted high levels of IL-21 upon antigen stimulation, suggesting they might contribute to humoral responses in these patients.



**Figure 2. Primary mDCs from controller neutralizers efficiently induce Tfh-like cells in vitro.** (A) Flow cytometry analysis of CXCR5 and PD-1 expression on naive CD4<sup>+</sup> T cells from an HIV-1-negative donor cultured for 7 days in the presence of media alone (left) or autologous naive B cells in the absence (middle) or the presence (right) of allogeneic mDCs isolated from an additional HIV-1-negative donor. Rectangles highlight CXCR5<sup>+</sup> CD4<sup>+</sup> T cells expressing either low or high levels of PD-1 (PD-1<sup>Lo</sup> and PD-1<sup>Hi</sup>, respectively). Dot plots from a representative experiment are shown. (B) Intracellular Bcl-6 expression in the indicated CD4<sup>+</sup> T cell subsets after coculture with mDCs for 7 days. Left panel shows dot plots from one representative experiment. Right panel shows cumulative mean fluorescence intensity of Bcl-6 data from *n* = 15 HIV-1-negative study subjects. Statistical significance was calculated using a Friedman test followed by Dunn's test (\*\**P* < 0.01; \*\*\**P* < 0.001). (C) Proportions of PD-1<sup>Hi</sup> (left) and PD-1<sup>Lo</sup> (right) CXCR5<sup>+</sup> CD4<sup>+</sup> T cells differentiated from naive CD4<sup>+</sup> T cells after 7 days of culture in the presence of autologous naive B cells (-) alone or in combination with allogeneic mDCs from HIV-1-negative donors (NG; *n* = 16), controller non-neutralizers (NN; *n* = 16), neutralizers (NT; *n* = 20), untreated (CP; *n* = 14) and HAART-treated (H; *n* = 14) chronic progressors. Statistical significance between mDCs from neutralizers and other conditions was calculated using a Kruskal-Wallis test followed by a Dunn's test (\**P* < 0.05; \*\*\**P* < 0.001). (D) Ratios of PD-1<sup>Lo</sup> versus PD-1<sup>Hi</sup> CXCR5<sup>+</sup> CD4<sup>+</sup> T cells differentiated in the presence of allogeneic mDCs from different study cohorts. Statistical significance between mDCs from NT versus other conditions was calculated using a Kruskal-Wallis test followed by a Dunn's test (\*\**P* < 0.01).

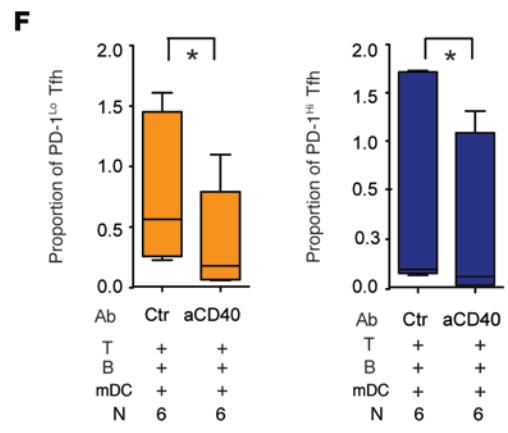
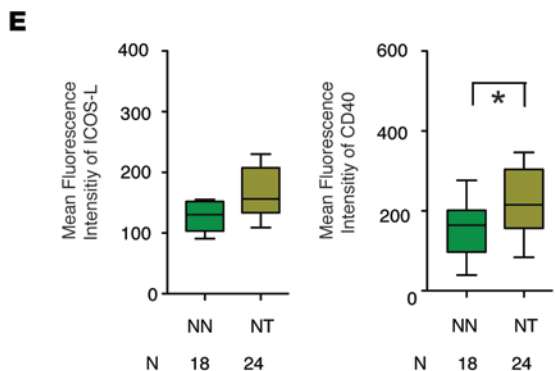
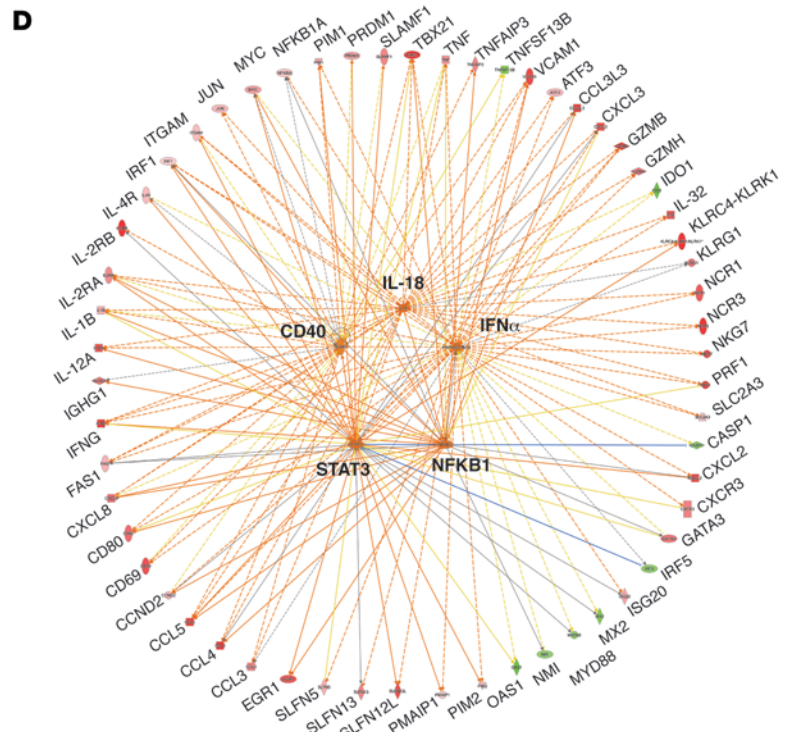
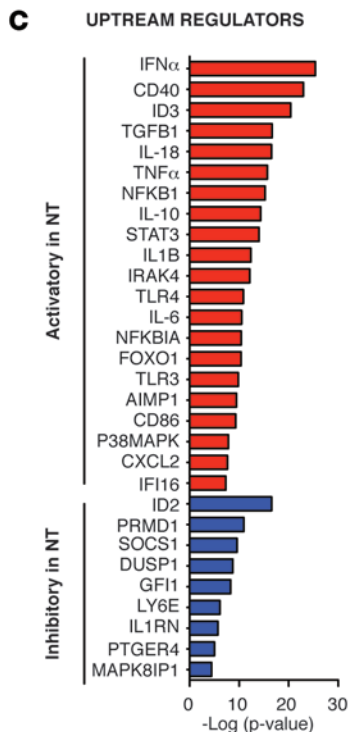
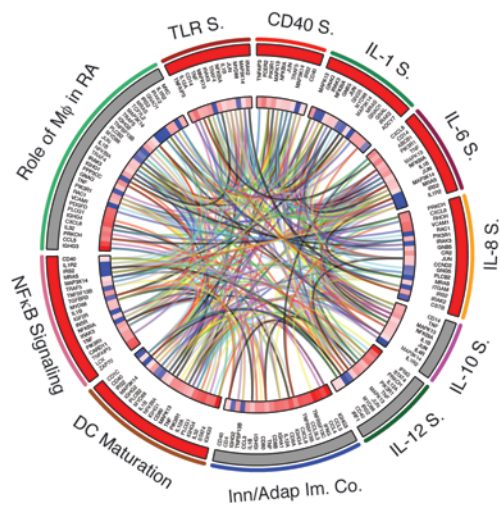
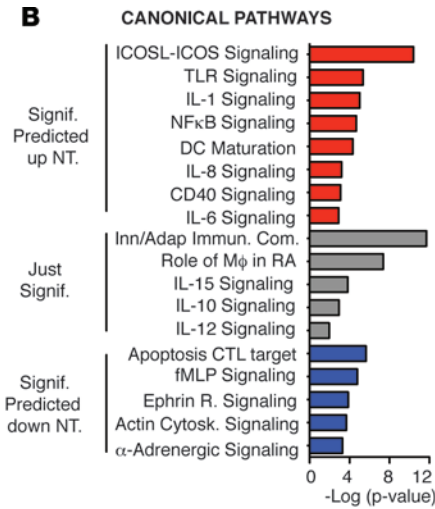
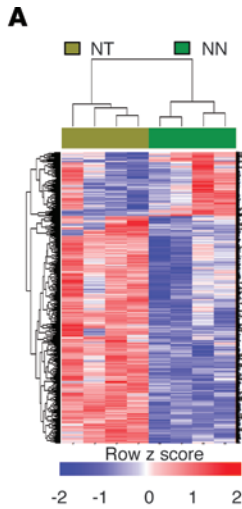
## Results

*Ratios of circulating PD-1<sup>Lo</sup>/PD-1<sup>Hi</sup> CXCR3<sup>+</sup> Tfh-like cells correlate with neutralizing antibody breadth in controllers.* Circulating CXCR5<sup>+</sup> CD4<sup>+</sup> T cells can be classified into CXCR3<sup>+</sup> and CXCR3<sup>-</sup> subsets (Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.89574DS1>). In untreated HIV-1 patients with progressive disease, evolution of broadly neutralizing antibody responses has been associated with proportions of circulating CXCR5<sup>+</sup>CXCR3<sup>-</sup>PD-1<sup>+</sup> Tfh-like

cells, which seem to represent the peripheral counterpart of conventional Tfh cells located in lymphoid tissues and are therefore termed pTfh (16). However, cellular immune responses supporting the evolution of bnAbs under conditions of limited antigen exposure are unknown. To address this, we selected a cohort of controllers who spontaneously maintained HIV-1 replication levels of less than 1,500 copies/ml and further subdivided them into 2 groups based on the presence (neutralizers) or absence (non-neutralizers) of antibodies with neutralizing activity against tier 2 HIV-1 viruses in plasma, with no significant difference in viral loads, CD4<sup>+</sup> T cell counts, and HLA allele distributions (Supplemental Tables 1 and 2). We found that in HIV-1 controller neutralizers, the proportions of CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>+</sup> pTfh cells were significantly lower than the proportions of CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>+</sup> CD4<sup>+</sup> T cells (Figure 1A). In addition, in contrast to prior studies in HIV-1 progressors (16), the proportions of CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>+</sup> pTfh cells were not associated with the breadth of neutralizing antibodies in controllers ( $P = 0.49$ ,  $r = 0.14$ ). Similarly, proportions of CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>+</sup> cells were also unrelated to neutralizing antibody breadth in this patient population ( $P = 0.76$ ,  $r = 0.06$ ). Based on the intensity of PD-1 surface expression, we further classified circulating CXCR5<sup>+</sup> T cells into PD-1<sup>hi</sup> and PD-1<sup>lo</sup> cells (Supplemental Figure 1B), as previously suggested (16). Interestingly, we found that proportions of both CXCR3<sup>+</sup> and CXCR3<sup>+</sup>PD-1<sup>lo</sup> cells in the peripheral blood were higher in controller neutralizers compared with controller non-neutralizers, while CXCR3<sup>+</sup> and CXCR3<sup>+</sup>PD-1<sup>hi</sup> cell subsets were not different between these 2 patient populations (Figure 1B). Yet proportions of neither PD-1<sup>lo</sup> nor PD-1<sup>hi</sup> CXCR5<sup>+</sup> CD4<sup>+</sup> T cells were associated with neutralizing antibody breadth in controller neutralizers, independent of CXCR3 expression (data not shown). In contrast, the ratios between PD-1<sup>lo</sup> and PD-1<sup>hi</sup> cells within CXCR5<sup>+</sup> CXCR3<sup>+</sup> T cells were positively correlated with the breadth of the HIV-1-specific neutralizing antibody responses (Figure 1C, nominal  $P = 0.02$ ) in controller neutralizers. These associations seemed to be specific of the CXCR5<sup>+</sup>CXCR3<sup>+</sup> CD4<sup>+</sup> T cell compartment, since the ratio of PD-1<sup>lo</sup> to PD-1<sup>hi</sup> cells within the CXCR5<sup>+</sup>CXCR3<sup>+</sup> CD4<sup>+</sup> T cell populations was unrelated to neutralizing antibody breadth (Figure 1C). Together, our data indicate that enrichment of CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> relative to CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>hi</sup> Tfh-like cells in blood is associated with the breadth of neutralizing antibodies in the absence of high-level HIV-1 replication.

*Primary myeloid DCs from HIV-1 controller neutralizers preferentially prime naive CD4<sup>+</sup> T cells into PD-1<sup>lo</sup> Tfh-like cells.* A number of experimental animal studies suggest that DCs are indispensable for initiating a Tfh development program in naive CD4<sup>+</sup> T cells (11, 12). Therefore, we hypothesized that the relative enrichment of PD-1<sup>lo</sup> versus PD-1<sup>hi</sup> CXCR3<sup>+</sup> Tfh-like cells in the blood from controller neutralizers might be associated with differential priming by DCs. To evaluate the ability of human myeloid DCs (mDCs) to polarize CD4<sup>+</sup> T cells toward Tfh lineage commitment in vitro, we established a coculture system with autologous naive CD4<sup>+</sup> T cells and naive B cells from HIV-1-negative individuals in the presence or absence of primary mDCs isolated from the blood of an allogeneic healthy donor, without addition of exogenous antigens. After 7 days of coculture, maturation of naive CD4<sup>+</sup> T cells into cells with a Tfh-like phenotype was analyzed by flow cytometry. As shown in Figure 2A, and consistent with previous studies (25), CD4<sup>+</sup> T cells were unable to efficiently upregulate the expression of the Tfh markers CXCR5 and PD-1 when cultured in media or in the presence of naive B cells alone. However, when mDCs were added to the coculture, a significant proportion of CXCR5<sup>+</sup>PD-1<sup>+</sup> T cells was detected on day 7 of culture (Figure 2A), suggesting de novo differentiation of naive CD4<sup>+</sup> T cells into Tfh-like cells in vitro. Similar to cells from peripheral blood, CXCR5<sup>+</sup> T cells induced in this assay could also be subdivided into PD-1<sup>lo</sup> and PD-1<sup>hi</sup> subpopulations (Figure 2A). Further phenotypical analysis revealed that both PD-1<sup>lo</sup> and PD-1<sup>hi</sup> CXCR5<sup>+</sup> T cells also expressed additional Tfh markers such as Bcl-6 and ICOS (26, 27) (Figure 2B and Supplemental Figure 2A), while expression of FoxP3, a marker for follicular regulatory T (Tfr) cells (28), was low (Supplemental Figure 2A). Of note, most DC-primed CXCR5<sup>+</sup>PD-1<sup>+</sup> T cells expressed the chemokine receptor CXCR3 (Supplemental Figure 2A). To examine whether the primed T cells generated in our coculture system were more similar to Th1 or Tfh1 cells, we analyzed intracellular expression levels of the Th1 transcription factor Tbet (29). As shown in Supplemental Figure 2, A and B, Tbet was detected in CXCR5<sup>+</sup>PD-1<sup>+</sup> T cells, but levels of expression were much lower than in bona fide Th1 cells primed with LPS-pulsed mDCs (Supplemental Figure 2B). Therefore, our data indicate that human primary mDCs are required to efficiently prime naive CD4<sup>+</sup> T cells into CXCR5<sup>+</sup>PD-1<sup>+</sup> Tfh1-like cells in the presence of B cells in vitro. Importantly, in vitro generation of CXCR3<sup>+</sup>ICOS<sup>+</sup> PD-1<sup>lo</sup> and PD-1<sup>hi</sup> Tfh1-like cells was also observed in the presence of primary mDCs isolated from lymph node biopsies from HIV-1-negative donors (Supplemental Figure 2C).





**Figure 3. Transcriptional analysis of DCs from HIV-1 controller neutralizers.** (A) Heatmap representing 930 genes differentially expressed (FDR-adjusted  $P < 0.05$ ) between peripheral blood mDCs from neutralizers (NT) and non-neutralizers (NN) ( $n = 4$  patients in each group). (B) Left panel: Canonical pathways significantly (Signif.) upregulated (red) and downregulated (blue) (Fisher's exact test,  $-\log_{10} P$  values for each represented pathways are shown) in transcriptional signatures in mDCs from NT, as predicted by Ingenuity Pathway Analysis (IPA). Pathways in gray are significant but without known degree of functional activation/inhibition. Right panel: Circos plot representing genes shared between pathways differentially expressed in mDCs from NT. Inner circle reflects the  $\log_2$  fold change in gene expression intensity between NT and NN. Pathways predicted to be activated in NT are highlighted in red in the outer circle; pathways in gray are significant but with unknown degree of functional activation. Inn/Adap Immun. Com., innate and adaptive immune communication; Cytosk., cytoskeleton; Ephrin R., ephrin receptor; S., signaling. (C) Significant putative upstream regulators with predicted activating (red) or inhibitory (blue) influence on transcriptional signatures in mDCs from NT, as determined by IPA. (D) Predicted network interactions of 5 putative upstream regulators of transcriptional signatures in mDCs from NT. Activating impulses are highlighted in orange; inhibitory signals are marked in blue. Target genes upregulated in mDCs from NT are labeled in red; downregulated genes are labeled in green. (E) Mean fluorescence intensity of surface levels of ICOS ligand (ICOS-L) and CD40 assessed by flow cytometry in gated mDCs from HIV-1 controller NN ( $n = 18$ ) and NT ( $n = 24$ ). Statistical significance was calculated using a 2-tailed Mann-Whitney  $U$  test ( $*P < 0.05$ ). (F) Changes in proportions of PD-1<sup>lo</sup> (left) and PD-1<sup>hi</sup> (right) Tfh-like cells generated in the presence or absence of anti-CD40 blocking antibodies ( $n = 6$  experiments). Differences were tested for significance using a 2-tailed Wilcoxon matched-pairs signed rank test ( $*P < 0.05$ ).

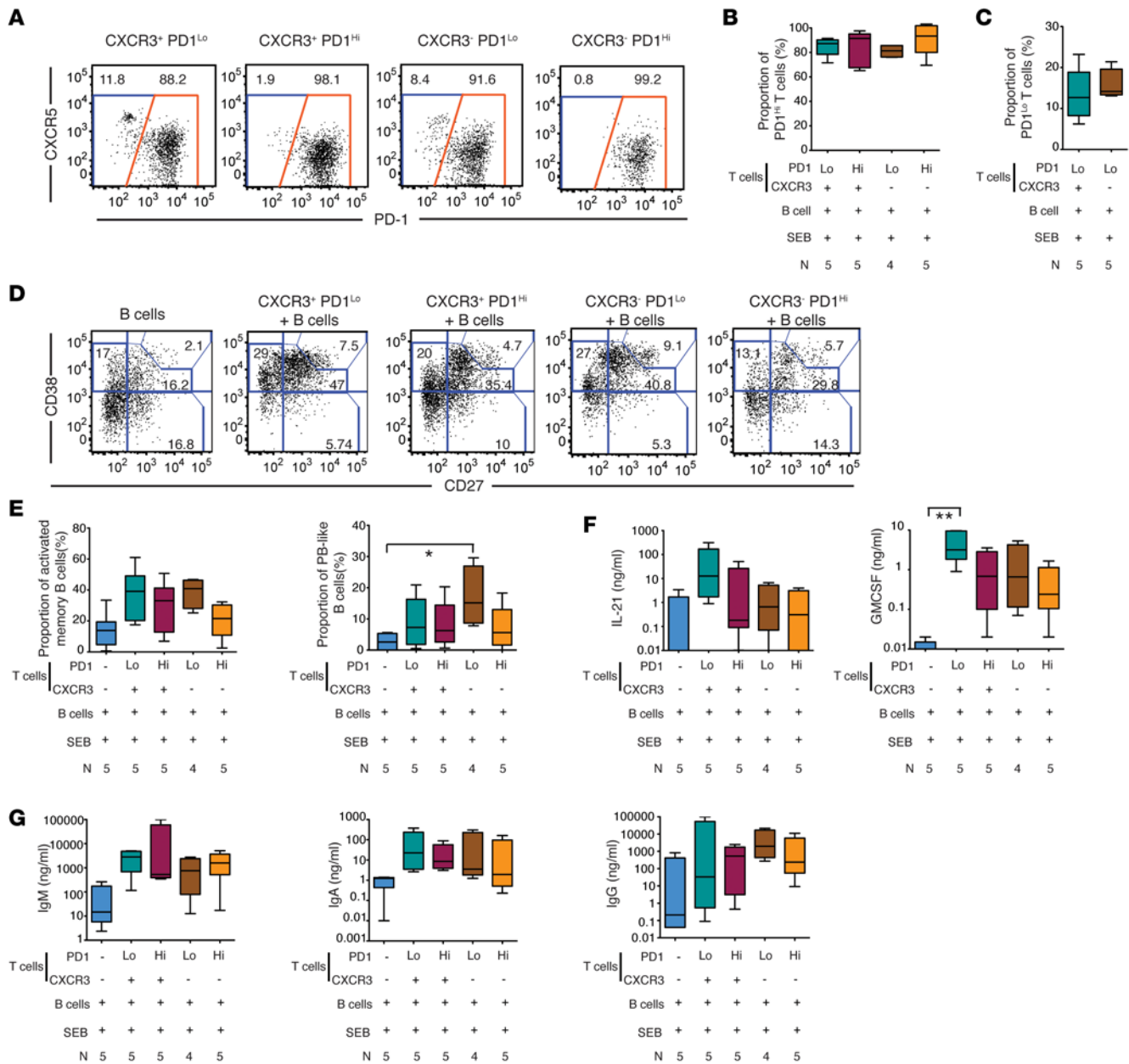
Using this experimental system, we next determined the ability of mDCs from different cohorts of HIV-1-infected individuals to prime Tfh-like cells in vitro. To this end, we evaluated the Tfh-priming potential of mDCs from controller neutralizers (NT), non-neutralizers (NN), and viremic (CP) and highly active antiretroviral therapy-treated (HAART-treated; H) chronically HIV-1-infected individuals. A cohort of HIV-1-negative persons (NG) was also included for comparative purposes. Subsequently, mDCs were isolated from all study groups, and their in vitro Tfh priming abilities were tested by coculture assays using identical allogeneic naive CD4<sup>+</sup> T and B cells from HIV-1-negative donors. As shown in Figure 2C, generation of PD-1<sup>hi</sup> Tfh-like cells was less efficient in the presence of mDCs from HIV-1-infected donors compared with uninfected individuals, but mDCs from controller neutralizers and non-neutralizers seemed equally effective in inducing cells with a PD-1<sup>hi</sup> Tfh phenotype. However, mDCs from controller neutralizers induced higher proportions of PD-1<sup>lo</sup> Tfh-like cells compared with controller non-neutralizers and chronically infected, viremic, or HAART-treated individuals (Figure 2C). Notably, the ratios of PD-1<sup>lo</sup> to PD-1<sup>hi</sup> Tfh-like cells generated in the presence of mDCs from controller neutralizers were higher than in assays with mDCs from all other study cohorts, and significantly exceeded the ratios of PD-1<sup>lo</sup> to PD-1<sup>hi</sup> cells generated in the presence of healthy individuals (Figure 2D). Notably, preferential induction of CXCR5<sup>+</sup>PD-1<sup>lo</sup> T cells by mDCs from HIV-1 controller neutralizers was not associated with significantly higher levels of specific cytokines (Supplemental Figure 2D), despite a tendency for higher detection of IL-6, IL-10, and IL-21. Similarly, cultures performed with mDCs from controller neutralizers did not show specific phenotypic alterations in cocultured B cells (Supplemental Figure 3, A and B) or significant changes in the Ig classes secreted by B cells (Supplemental Figure 3C). However, the contribution of class G Ig to the total amount of secreted Ig was highest in assays with mDCs from controller neutralizers (Supplemental Figure 3C). Together, these data indicate that primary mDCs from controller neutralizers have superior abilities to prime PD-1<sup>lo</sup>CXCR3<sup>+</sup> CXCR5<sup>+</sup> Tfh-like cells in vitro, compared with mDCs from controller non-neutralizers.

*Distinct transcriptional signatures in mDCs from controller neutralizers.* To better understand molecular mechanisms underlying enhanced abilities of mDCs from controller neutralizers to prime Tfh-like cells, we analyzed transcriptional profiles of mDCs from 4 controller neutralizers compared with cells from 4 controller non-neutralizers. These analyses revealed significant differences (FDR-adjusted  $P < 0.05$ ) in the expression of 930 genes in mDCs from controller neutralizers (Figure 3A and Supplemental Table 3), as determined by DESeq2 (30). Overall, pathways upregulated in controller neutralizers shared a high number of genes with interconnected functions in innate and adaptive immunity, consistent with the diverse role of DCs in orchestrating antiviral immune defense (Figure 3B). Interestingly, we observed that pathways previously associated with Tfh priming, such as ICOS-L-ICOS interactions ( $P = 3.98 \times 10^{-11}$ ) and CD40-dependent signaling ( $P = 0.00079$ ) (31) (Figure 3B), were enriched in transcriptional signatures of mDCs from controller neutralizers. In addition, genes encoding for signaling components of the IL-6 ( $P = 0.001$ ) and IL-1 ( $P = 9.54 \times 10^{-6}$ ) pathways, critical for functional development of Tfh cells and the inhibition of alternative T helper cell lineages (32, 33), were also significantly upregulated in mDCs from controller neutralizers (Figure 3B), as were transcripts involved in general DC maturation and activation, features of mDCs previously involved in improved Tfh generation (34). Consistent with these observations, biocomputational analysis identified signaling through CD40 ( $P = 1.05 \times 10^{-23}$ ) or CD86 ( $P = 5.05 \times 10^{-10}$ ); secretion of multiple effector cytokines such as IFN- $\alpha$  ( $P = 3.98 \times 10^{-26}$ ), TNF- $\alpha$

( $1.96 \times 10^{-16}$ ), IL-1 $\beta$  ( $P = 4.55 \times 10^{-13}$ ), IL-6 ( $P = 3.2 \times 10^{-11}$ ), IL-10 ( $P = 4.5 \times 10^{-15}$ ), and IL-18 ( $P = 2.89 \times 10^{-17}$ ) (Figure 3C); and transcription factors and signal mediators such as NFKB1 ( $P = 5.88 \times 10^{-16}$ ), STAT3 ( $P = 9.47 \times 10^{-15}$ ), IRAK4 ( $P = 7.45 \times 10^{-13}$ ), and ID3 ( $P = 3.97 \times 10^{-21}$ ) as putative upstream regulators of transcriptional signatures in mDCs from controller neutralizers (Figure 3, C and D). In contrast, transcriptional pathways downregulated in DC from controller neutralizers had no immediate connection to Tfh priming and DC function (Figure 3B). To further explore these observations, we performed functional in vitro coculture assays between DCs and T/B cells using a Transwell experimental system. As previously described for CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>+</sup> cells (31, 35), in vitro priming of CXCR3<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells seemed to require direct contact with mDCs (Supplemental Figure 4A). Flow cytometry analysis revealed that among all costimulatory molecules tested, ICOS-L and more significantly CD40 tended to be expressed at higher levels on mDCs from controller neutralizers (Figure 3E and Supplemental Figure 4B), consistent with the described transcriptional profiling experiments. Importantly, inhibition of cellular communication via CD40/CD40L (Figure 3F) significantly abrogated the priming of Tfh-like cells by mDCs, although such interactions alone did not explain the preferential induction of PD-1<sup>lo</sup> by mDCs from controller neutralizers. Collectively, these data indicate that mDCs from controller neutralizers are characterized by transcriptional signatures associated with key pathways involved in Tfh priming.

*CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> CD4<sup>+</sup> T cells are detectable in lymphoid tissues in vivo.* We next investigated the presence of CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells in peripheral blood and tissue samples isolated directly ex vivo in samples from HIV-1–negative persons (Supplemental Figure 1, A and B). We found that as in peripheral blood, the proportions of CXCR5<sup>+</sup>CXCR3<sup>+</sup> CD4<sup>+</sup> T cells approximated those of CXCR5<sup>+</sup>CXCR3<sup>+</sup> CD4<sup>+</sup> T cells in the lymph node, and that the majority of CXCR5<sup>+</sup> CD4<sup>+</sup> T cells in these tissues were PD-1<sup>lo</sup>, independent of CXCR3 coexpression (Supplemental Figure 1, A and B). In contrast, in tonsil tissue, PD-1<sup>lo</sup> cells were mostly detected in CXCR5<sup>+</sup>CXCR3<sup>+</sup> cells, while CXCR5<sup>+</sup>CXCR3<sup>+</sup> CD4<sup>+</sup> T cells consisted almost entirely of PD-1<sup>hi</sup> cells. Notably, CXCR3<sup>+</sup>PD-1<sup>hi</sup> cells were the dominant cell subset within CXCR5<sup>+</sup> lymphocytes in tonsils, while CXCR3<sup>+</sup>PD-1<sup>lo</sup> cells represented the largest cell compartment within CXCR5<sup>+</sup> CD4<sup>+</sup> T cells from blood and lymph nodes (Supplemental Figure 1B). Moreover, within lymph nodes and tonsils, Bcl-6 was upregulated in all CXCR5<sup>+</sup> CD4<sup>+</sup> T cells, irrespective of their CXCR3 or PD-1 expression levels, as opposed to CXCR5<sup>+</sup> CD4<sup>+</sup> T cells from peripheral blood, in which Bcl-6 levels were very low and indistinguishable from those of total CD4<sup>+</sup> T cells, consistent with prior results (3) (Supplemental Figure 5). ICOS was more strongly expressed in PD-1<sup>hi</sup> Tfh-like cells compared with PD-1<sup>lo</sup> cells across all tissues (Supplemental Figure 5). Together, our data indicate that CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> cells are present in vivo in lymphoid tissues.

*CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells support B cell activation and differentiation.* Previous studies defined CXCR3<sup>+</sup> pTfh cells as poor supporters of B cell maturation based on their weak ability to stimulate class switching to IgG in B cells in vitro (15, 16). However, our results suggest that the presence of CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells might be beneficial for the development of neutralizing antibodies against HIV-1 in controllers. To better understand the functional characteristics of CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells, different pTfh cell subsets from the blood of HIV-negative individuals (Supplemental Figure 1, A and B) were sorted and cultured with autologous total B cells in the presence of staphylococcal endotoxin B (SEB). After 6 days of culture, differentiation of pTfh subsets, maturation of B cells, cytokine secretion profiles, and levels of secreted Igs were analyzed. As shown in Figure 4, A and B, most CXCR3<sup>+</sup> and CXCR3<sup>+</sup> CXCR5<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells differentiated into PD-1<sup>hi</sup> Tfh-like cells in the presence of antigens and autologous B cells; however, some of the CXCR3<sup>+</sup> and CXCR3<sup>+</sup> PD-1<sup>lo</sup> Tfh-like cells seemed to be able to retain their original PD-1<sup>lo</sup> phenotype during coculture (Figure 4, A and C). In addition, substantial levels of proliferation were detected on all subsets of pTfh cells present on day 6 of culture (Supplemental Figure 8A). Coculture of B cells with any of the pTfh cell subsets was accompanied by a tendency for decreased proportions of resting memory B cells (Figure 4D and Supplemental Figure 6A), and a marked upregulation of CD38 and CD27, leading to elevated frequencies of CD38<sup>int</sup>CD27<sup>int</sup> activated memory B cells, CD38<sup>hi</sup>CD27<sup>hi</sup> plasmablast-like B cells (Figure 4, D and E), and CD38<sup>hi</sup>CD27<sup>+</sup> transitional B cells (Supplemental Figure 6A). Notably, PD-1<sup>lo</sup> pTfh cells tended to be more efficient than PD-1<sup>hi</sup> cells in inducing activated memory B cells (Figure 4E). Overall, PD-1<sup>lo</sup> and PD-1<sup>hi</sup> CXCR3<sup>+</sup> pTfh cells were comparable to CXCR3<sup>+</sup> pTfh subsets in the extent to which they induced phenotypic maturation in B cells. Interestingly, PD-1<sup>lo</sup>CXCR3<sup>+</sup> pTfh cells seemed to secrete higher levels of IL-21 and, more significantly, of GM-CSF, IL-6, and IL-10 in a 6-day coculture assay



**Figure 4. CXCR3<sup>+</sup>PD-1<sup>lo</sup> pTfh cells support B cell activation and differentiation.** (A) Dot plots reflect CXCR5 versus PD-1 expression on sorted CXCR3<sup>+</sup> and CXCR3<sup>-</sup> PD-1<sup>lo</sup> and PD-1<sup>hi</sup> pTfh cells on day 6 in coculture with autologous total B cells in the presence of SEB in a representative experiment. Numbers above the gates represent percentages of cells. (B and C) Proportions of PD-1<sup>hi</sup> (B) and PD-1<sup>lo</sup> (C) CD4 T cells after 6-day culture of isolated CXCR3<sup>+</sup>PD-1<sup>lo</sup> (*n* = 5), CXCR3<sup>+</sup>PD-1<sup>hi</sup> (*n* = 5), CXCR3<sup>-</sup>PD-1<sup>lo</sup> (*n* = 4), and CXCR3<sup>-</sup>PD-1<sup>hi</sup> (*n* = 5) cells with autologous B cells and SEB. (D) Representative flow cytometry analysis showing CD38 vs CD27 surface expression on gated CD19<sup>+</sup> B cells on day 6 of coculture with SEB alone or the indicated pTfh populations. Numbers within the gates represent percentages of cells. (E) Proportions of CD38<sup>int</sup>CD27<sup>int</sup> activated memory and CD38<sup>hi</sup>CD27<sup>hi</sup> plasmablast-like (PB-like) B cells on day 6 of coculture with the indicated pTfh populations. CXCR3<sup>+</sup>PD-1<sup>lo</sup> (*n* = 5), CXCR3<sup>+</sup>PD-1<sup>hi</sup> (*n* = 5), CXCR3<sup>-</sup>PD-1<sup>lo</sup> (*n* = 4), and CXCR3<sup>-</sup>PD-1<sup>hi</sup> (*n* = 5). Differences between each condition with indicated pTfh subset and control B cells activated with SEB alone were tested for statistical significance using a Kruskal-Wallis test followed by Dunn's test (\**P* < 0.05). (F and G) Luminex analysis of the indicated cytokine (F) and Ig (G) concentrations present in supernatants on day 6 in the indicated cocultures with CXCR3<sup>+</sup>PD-1<sup>lo</sup> (*n* = 5) CXCR3<sup>+</sup>PD-1<sup>hi</sup> (*n* = 5), CXCR3<sup>-</sup>PD-1<sup>lo</sup> (*n* = 4), and CXCR3<sup>-</sup>PD-1<sup>hi</sup> (*n* = 5) pTfh cells. Statistical significance was calculated using a Kruskal-Wallis test followed by a Dunn's test (\*\**P* < 0.01).

with total B cells and SEB (Figure 4F and Supplemental Figure 6B). In agreement with previous studies, only CXCR3<sup>-</sup> pTfh cells were able to efficiently induce full class switching to IgG in B cells, dominated by IgG1 (Figure 4G and Supplemental Figure 6C) (16–18). In contrast, PD-1<sup>lo</sup> and PD-1<sup>hi</sup> CXCR3<sup>+</sup> cells seemed capable of stimulating B cells to produce IgM, IgA, and IgG3, but not IgG1, as previously reported (16–18), suggesting a more limited ability of these pTfh subsets to support Ig class switching to IgG in our

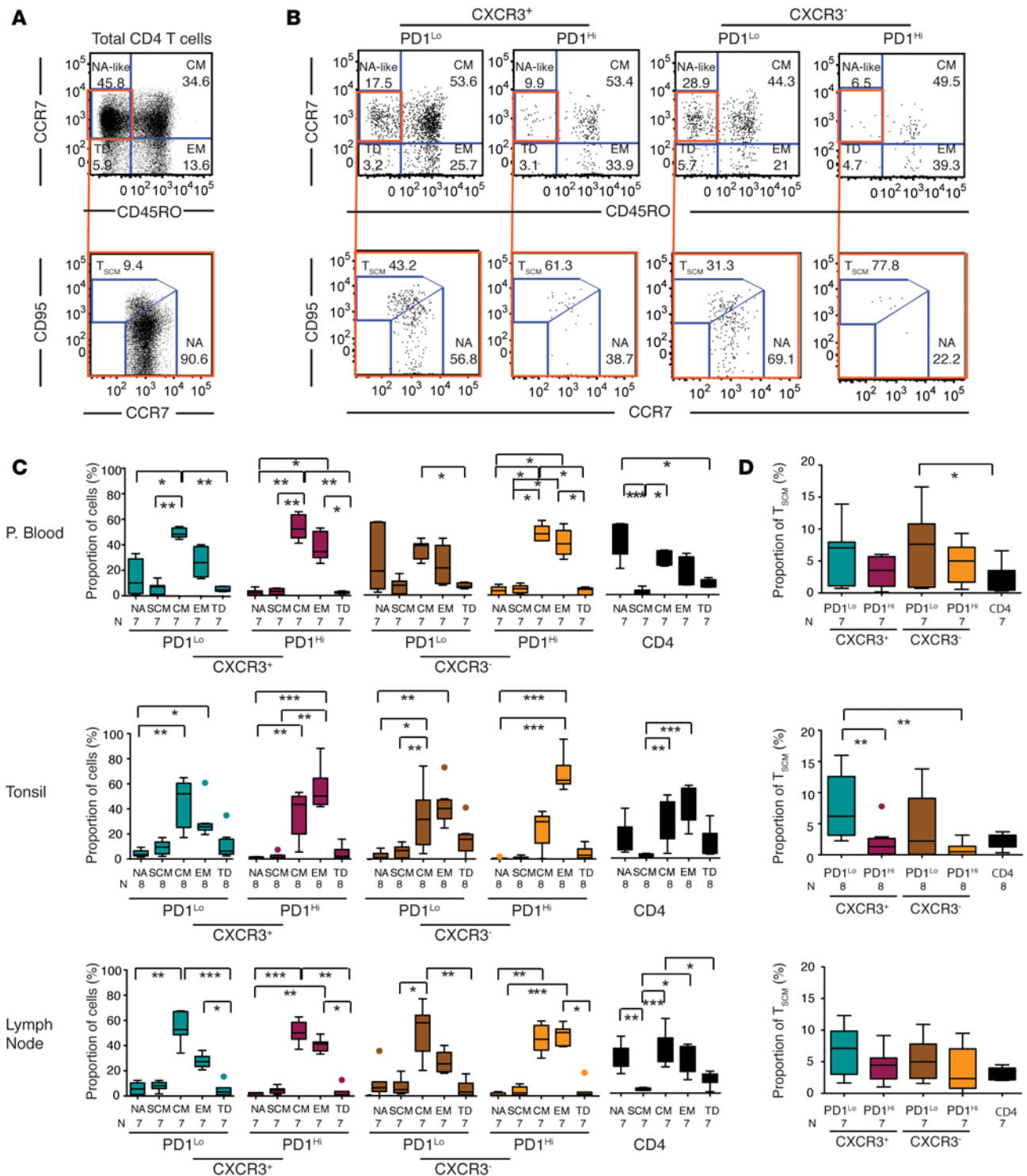


6-day coculture assays (Figure 4G). Together, these data indicate that both CXCR3<sup>+</sup> and CXCR3<sup>-</sup> PD-1<sup>lo</sup> pTfh subsets mature into PD-1<sup>hi</sup> cells upon Ag stimulation, and that CXCR3<sup>+</sup> pTfh cells display distinct but partially overlapping functional abilities to support B cells compared with conventional CXCR3<sup>-</sup> pTfh cells. These findings suggest that both CXCR3<sup>+</sup> and CXCR3<sup>-</sup> pTfh cells might participate in the development of Ab breadth at different levels.

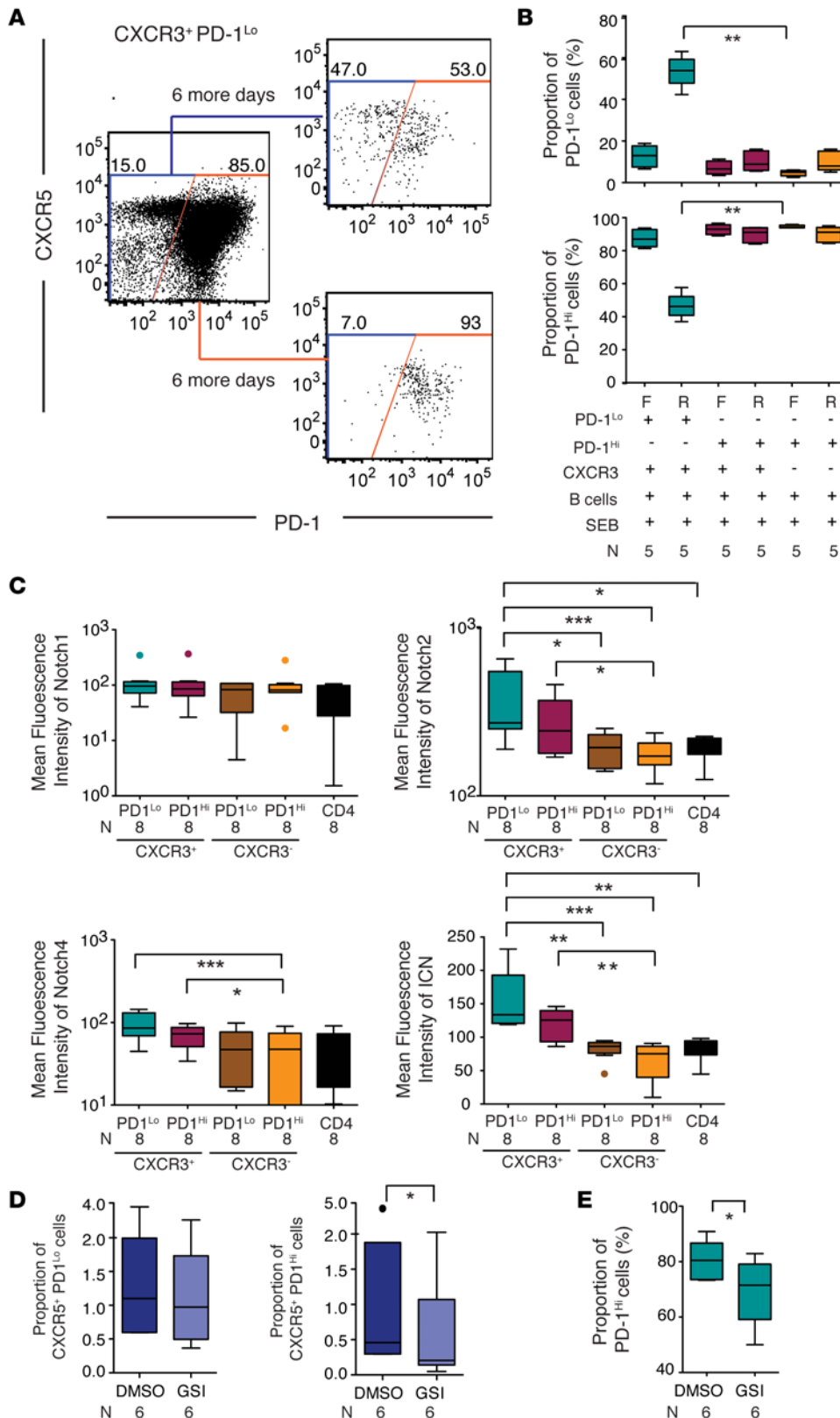
*CXCR3<sup>+</sup>PD-1<sup>lo</sup> pTfh cells differentiate into PD-1<sup>hi</sup> cells in a Notch-dependent fashion.* While most PD-1<sup>lo</sup> Tfh-like cells differentiated into PD-1<sup>hi</sup> upon antigenic stimulation, we observed a small proportion of PD-1<sup>lo</sup> Tfh-like cells that were able to retain their original CXCR5<sup>+</sup>PD-1<sup>lo</sup> phenotype during coculture with B cells and SEB, suggesting self-renewal or homeostatic proliferation. To test this possibility, we first analyzed the expression of memory cell markers (Figure 5, A and B) on different Tfh-like subsets from human blood and lymphoid tissues. As shown in Figure 5C, PD-1<sup>lo</sup> and PD-1<sup>hi</sup> cells from both CXCR3<sup>+</sup> and CXCR3<sup>-</sup> peripheral Tfh cells were dominated by CCR7<sup>+</sup>CD45RO<sup>+</sup> central memory T cells; in contrast, total CD4<sup>+</sup> T cells were dominated by naive cells. In tonsils and lymph nodes, CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> CD4<sup>+</sup> T cells also preferentially exhibited a central memory phenotype, while PD-1<sup>hi</sup> cells, specifically within the CXCR3<sup>-</sup> T cell pools, included higher proportions of CCR7<sup>-</sup>CD45RO<sup>+</sup> effector memory cells (Figure 5C). Importantly, we observed that PD-1<sup>hi</sup> cells from all tissue compartments consisted mostly of CD45RO<sup>+</sup> cells, while both CXCR3<sup>+</sup> and CXCR3<sup>-</sup> PD-1<sup>lo</sup> Tfh populations contained larger and more distinct populations of CD45RO<sup>-</sup> naive-like cells (Figure 5B). Subsequent experiments demonstrated that many of these CD45RO<sup>-</sup> Tfh cells expressed CD95, a memory cell marker that when expressed on otherwise naive-appearing CD4<sup>+</sup> T cells defines a highly immature population of extremely long-lived memory CD4<sup>+</sup> T cells with stem cell-like properties (Figure 5B) (36). In fact, in contrast to total CD4 T cells (Figure 5D), a large proportion of naive-like cells present in the different CXCR5<sup>+</sup> subsets from the analyzed tissue compartments exhibited a CD95<sup>+</sup> T stem cell memory phenotype, and the relative proportions of these CD45RO<sup>-</sup>CD95<sup>+</sup> cells tended to be higher within the PD-1<sup>lo</sup> populations (Figure 5D). Importantly, a similar memory subset distribution was observed between circulating Tfh-like cell populations from HIV-1 controller neutralizers and non-neutralizers (Supplemental Figure 7A). Interestingly, frequencies of stem cell memory T cells within the CXCR3<sup>+</sup>PD-1<sup>lo</sup> cell populations appeared to be increased in HIV-1 controller neutralizers (Supplemental Figure 7B). Therefore, CXCR5<sup>+</sup>PD-1<sup>lo</sup> CD4<sup>+</sup> T cells seemed to contain higher proportions of more immature stem cell memory T cells.

To investigate stem cell-like precursor properties of PD-1<sup>lo</sup> Tfh-like cells, we analyzed the developmental fate of CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> in serial coculture experiments. For this purpose, CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> cells that maintained their original phenotype during an initial 6-day coculture assay with B cells and SEB were sorted and exposed to 6 additional days of culture with B cells and SEB. Interestingly, a considerable proportion of sorted CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> CD4<sup>+</sup> T cells that remained PD-1<sup>lo</sup> during the initial culture also retained their original phenotype during secondary culture, although a proportion of these cells was able to differentiate into PD-1<sup>hi</sup> cells (Figure 6, A and B). In contrast, the phenotype of sorted PD-1<sup>hi</sup> pTfh cells remained unchanged after the primary and secondary cultures, independent of CXCR3 expression (Figure 6B). Importantly, cells capable of maintaining a PD-1<sup>lo</sup> phenotype during primary (Supplemental Figure 8, B and C) and secondary cultures (Supplemental Figure 8D) showed signs of active proliferation. This suggests that even though the majority of PD-1<sup>lo</sup> CXCR5<sup>+</sup> cells ultimately transition to PD-1<sup>hi</sup> cells, a proportion of these cells appear to maintain their original phenotype while proliferating, consistent with homeostatic self-renewal, a key component of a stem cell-like functional profile.

To investigate mechanisms that may dictate the ability of PD-1<sup>lo</sup> pTfh cells to repopulate PD-1<sup>hi</sup> pTfh cells, we focused on the Notch signaling cascade, which has previously been implicated in regulating the developmental fate of organ-specific stem cells (37, 38), memory T cells (39–41), and T follicular helper cells (42). We observed that Notch receptors 2 and 4, as well as the Notch active intracellular domain, were upregulated on CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> cells and, to a lesser extent, on CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>hi</sup> CD4<sup>+</sup> T cells from peripheral blood when compared with total CD4<sup>+</sup> T cells (Figure 6C). Moreover, inhibition of Notch signaling by gamma secretase inhibitors (GSI) in naive T cells cocultured with autologous naive B cells and allogenic mDCs significantly inhibited the generation of PD-1<sup>hi</sup> but not PD-1<sup>lo</sup> Tfh-like cells in vitro (Figure 6D). A similar inhibition in the generation of PD-1<sup>hi</sup> cells was observed when Notch signaling was blocked in sorted CXCR3<sup>+</sup>PD-1<sup>lo</sup> pTfh cells cocultured with autologous B cells and SEB (Figure 6E), suggesting that Notch signaling is critical in regulating differentiation of PD-1<sup>lo</sup>CXCR5<sup>+</sup> CD4<sup>+</sup> cells into PD-1<sup>hi</sup> cells. Together, these data suggest that circulating CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> CD4<sup>+</sup> T cells represent a distinct population of progenitor cells able to repopulate the PD-1<sup>hi</sup> effector pTfh cells in a Notch-dependent fashion.



**Figure 5. Characterization of memory cell subsets within Tfh-like cells.** (A and B) Representative flow cytometry analysis of memory cell subsets in total CD4<sup>+</sup> T cells (A) and in PD1<sup>Lo</sup> and PD1<sup>Hi</sup> CXCR3<sup>+</sup>CXCR3<sup>-</sup> and CXCR3<sup>-</sup>CXCR3<sup>+</sup> pTfh cells (B). CCR7 versus CD45RO expression defines central memory (CM), effector memory (EM), terminally differentiated (TD) and naive-like (NA-like) T cells (upper panels). NA-like T cells are subdivided into memory stem cells (SCM) and naive (NA) cells based on expression levels of CD95 (lower panels). (C and D) Proportions of memory subsets within PD1<sup>Lo</sup> and PD1<sup>Hi</sup> CXCR3<sup>+</sup> and CXCR3<sup>-</sup> cells, and total CD4<sup>+</sup> T cells (CD4) from peripheral blood (P. Blood; upper panel, n = 7), tonsils (middle panel, n = 8), and lymph nodes (lower panel, n = 7) (C). Proportions of T memory stem cells (SCM) within each pTfh subset from the indicated compartments are represented separately in D. Statistical significance within individual subset (C) and in T<sub>SCM</sub> proportions across different subsets (D) was calculated using a Friedman test followed by a Dunn's test (\*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001).



**Figure 6. CXCR3<sup>+</sup>PD-1<sup>lo</sup> pTfh cells can differentiate into PD-1<sup>hi</sup> cells in a Notch-dependent fashion.** (A) Sorted CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells were cultured with autologous B cells and SEB for 6 days (left plot). PD-1<sup>lo</sup> and PD-1<sup>hi</sup> events from these cultures were selectively resorted and cocultured for 6 additional days with B cells and SEB. Right plots show PD-1 expression on resorted PD-1<sup>lo</sup> and PD-1<sup>hi</sup> cells after the second 6-day culture assay. Dot plots from a representative experiment are shown. Numbers on the gates represent the proportion of cells. (B) Proportions of remaining PD-1<sup>lo</sup> and PD-1<sup>hi</sup> cells detected on day 6 of coculture of freshly sorted (F) or resorted (R) CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> (*n* = 5), CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>hi</sup> (*n* = 5), and CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>hi</sup> (*n* = 5) pTfh cells with total B cells and SEB. Statistical significance was calculated using a Friedman test followed by Dunn's test (\**P* < 0.05; \*\**P* < 0.01). (C) Mean fluorescence intensity of Notch 1, 2, and 4 and intracellular Notch (ICN) in pTfh subsets and total CD4<sup>+</sup> T cells (*n* = 8). Statistical significance was calculated using a Friedman test followed by a Dunn's test (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001). (D) Proportions of PD-1<sup>lo</sup> or PD-1<sup>hi</sup> Tfh-like cells generated upon coculture of naive CD4<sup>+</sup> T cells with autologous B cells and allogeneic mDCs in the presence of gamma secretase inhibitors (GSI) in comparison to 100 nM DMSO (*n* = 6). Statistical significance was calculated using a 2-tailed Wilcoxon matched-pairs signed-rank test (\**P* < 0.05). (E) Proportions of PD-1<sup>hi</sup> T cells derived from CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> CD4<sup>+</sup> T cells after 6 days of coculture with autologous B cells and SEB in the presence of GSI compared with DMSO (*n* = 6). Statistical significance was calculated using a two-tailed Wilcoxon matched-pairs signed-rank test (\**P* < 0.05).

## Discussion

It is highly likely that the long-lasting induction of HIV-1–specific bnAbs by vaccines will require support from Tfh or Tfh-like cells. In previous studies, peripheral CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>+</sup> cells were found to correlate strongly with the development of HIV-1–specific bnAbs in patients with progressive diseases (16); however, such patients had continuous high levels of HIV-1 replications and elevated levels of immune activation that hardly reflect conditions for immune induction reproducible in uninfected recipients of a prophylactic HIV-1 vaccine candidate. Here, we used HIV-1 controllers, a group of patients widely used for investigation of HIV-1 immune defense mechanisms in previous studies (43–45), as a model for studying HIV-1–specific antibodies with increased neutralizing breadth in the absence of high-levels of viremia. Our observations demonstrate that ratios between CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> and CXCR3<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>hi</sup> Tfh-like cells in blood from HIV-1 controllers correlated with increased neutralizing breadth of HIV-1 antibodies in this particular patient population. In contrast, peripheral CXCR5<sup>+</sup>CXCR3<sup>−</sup>CD4<sup>+</sup> T cells were unrelated to HIV-1–specific antibodies with broader neutralizing breadth in these patients, irrespective of PD-1 expression. Interestingly, CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells expressed levels of Tfh markers such as ICOS and Bcl-6 in lymphoid tissue and peripheral blood comparable to those in conventional CXCR5<sup>+</sup>CXCR3<sup>−</sup>PD-1<sup>hi</sup> Tfh/pTfh cells. Moreover, CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like cells effectively produced cytokines for stimulation of B cell development and were able to differentiate into PD-1<sup>hi</sup> Tfh-like cells, although they were inferior to CXCR3<sup>−</sup> Tfh-like cells in inducing B cell Ig class switching to IgG1 in our coculture assays. Together, these studies suggest that at least under conditions of low viral loads and immune activation, CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> cells may make important contributions to the induction and/or maintenance of HIV-1 antibody responses with broad neutralizing breadth.

DCs in HIV-1 controllers seem to have a distinct functional and phenotypic profile that can contribute to the induction of highly functional HIV-1–specific T cell responses. These abilities have been associated with an altered expression profile of immunoregulatory receptors (46) and with enhanced abilities for HIV sensing and innate immune recognition (47). However, the impact of DCs from these patients on Tfh development is still unclear, and investigations of DCs on induction of Tfh have mostly been limited to animal studies (11, 48, 49) or experiments with in vitro generated monocyte-derived DCs (MDDCs) (25, 33), rather than primary DCs. To our knowledge, this is the first study to analyze the effects of primary DCs on Tfh priming in a rare group of HIV-1 controllers who are able to generate broader neutralizing breadth in the absence of high-level viral replications. Our results demonstrate that when cocultured with B cells, mDCs from these patients have a preferential ability to induce Tfh-like cells with low expression of PD-1, and a chemokine receptor expression profile that includes coexpression of CXCR3 and CXCR5. Notably, we found that Tfh priming by mDCs from controllers seems to be associated with increased transcriptional expression of genes involved in functional DC activity and in Tfh priming. Interestingly, secretion of specific cytokines, such as IL-6, a cytokine with a recognized role in Tfh priming by DCs (33), tended to be higher in cultures with cells from neutralizers and was also activated in transcriptional signatures of mDCs from these patients. Moreover, enhanced cell contact–dependent interactions mediated by CD40 and possibly ICOS-L, which were expressed at higher levels in mDCs from controller neutralizers (Figure 3E) and have previously been implicated in Tfh generation (50), may play an important role in this context. Notably, in our experimental in vitro system, primary mDCs did not efficiently prime CXCR3<sup>−</sup>CXCR5<sup>+</sup> Tfh-like cells, suggesting that the priming of this subset is more independent of DCs or might represent a different stage of T cell differentiation. Collectively, our findings highlight the importance of mDCs as critical mediators of Tfh lineage commitment and suggest that manipulation of mDCs might be an attractive strategy to improve Tfh differentiation in future HIV-1 vaccine studies.

Unexpectedly, this study showed that the emergence of HIV-1 antibodies with broader neutralizing breadth in HIV-1 controllers was associated with Tfh-like cells expressing CXCR3, a surface marker denoting cells considered less efficient in supporting B cell Ig class switching in previous in vitro studies (15–18). While our observations confirm that CXCR3<sup>+</sup> Tfh-like cells are inferior to CXCR3<sup>−</sup> Tfh-like cells in supporting IgG1 class switching, we did demonstrate that CXCR3<sup>+</sup> Tfh-like cells have abilities to induce class switching to IgG3 and to alternative Ig subtypes, to secrete high levels of B cell–supporting cytokines, and to induce phenotypical maturation of B cells at equivalent levels as CXCR3<sup>−</sup> Tfh-like cells, at least when analyzed over a longer, 6-day co-incubation period with total B cells and SEB stimulation, and not just in a short-term culture assay lasting for 48 hours (15). Together, these results suggest that



CXCR5<sup>+</sup>CXCR3<sup>+</sup> Tfh-like cells can contribute to helping B cell responses, and that CXCR5<sup>+</sup>CXCR3<sup>-</sup> and CXCR5<sup>+</sup>CXCR3<sup>+</sup> pTfh cells might have distinct but partially overlapping roles for supporting B cells. Consistent with a role as circulating memory cells, CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> cells in HIV-1 controllers may primarily contribute to maintaining long-term survival of B cells producing more broadly cross-reactive antibodies, and not to the initial priming of such immune responses, which is likely to preferentially occur in lymph node germinal centers and may largely be independent of circulating Tfh-like cells. A preferential role of CXCR3<sup>+</sup> Tfh-like cells in maintaining or expanding preexisting B cell responses, but not in the initial priming of new antigen-specific B cells, was also hypothesized in alternative contexts associated with limited antigen exposure, such as in recipients of vaccines against influenza (19). In contrast, in HIV-1 progressors with continuously ongoing high-level viremia, maintenance of HIV-1-specific B cells may more significantly depend on the presence of elevated amounts of circulating viral antigens. Notably, our data do not exclude the possibility that CXCR3<sup>-</sup> pTfh cells, which were also significantly elevated in controller neutralizers (Figure 1B), participate individually or in collaboration with CXCR3<sup>+</sup> pTfh cells in generating or maintaining more broadly neutralizing antibody-secreting B cells in these patients.

A large number of studies suggest that circulating CXCR5<sup>+</sup> T cells are memory Tfh cells (3), but the dynamics of their development and long-term survival remain poorly understood. Our data suggest that phenotypically defined subsets of circulating CXCR5<sup>+</sup> CD4<sup>+</sup> T cells may not only differ by effector functions, but also involve a developmental and maturational aspect. A linear developmental hierarchy from long-lasting immature central memory and stem cell memory to short-lived effector memory cells is experimentally well validated within the CD8<sup>+</sup> T cell memory compartment (36), and has also been suggested to occur with CD4<sup>+</sup> T cells (51). The results presented here suggest that a similar developmental hierarchy also exists within the pools of circulating Tfh-like cells, and that PD-1<sup>lo</sup> Tfh cells represent a long-lived precursor cell population that can replenish the more mature and short-lived PD-1<sup>hi</sup> effector Tfh cells. Interestingly, these PD-1<sup>lo</sup> Tfh-like cells included a substantial proportion of CD45RO<sup>-</sup> naive-like T cells, a cell population that was excluded in prior studies (15–17) but includes a fraction of highly immature memory T cells with increased stem cell-like properties (36). The ability to maintain a larger proportion of PD-1<sup>lo</sup> memory Tfh-like cells that are enriched for a T memory stem cell phenotype relative to PD-1<sup>hi</sup> pTfh cells could represent a distinct aspect of HIV-1 controllers with more broadly neutralizing antibody responses, and appears to separate these patients from HIV-1 progressors, in whom high-level viremia may lead to more differentiated and possibly more exhausted PD-1<sup>hi</sup> pTfh cell populations (52). Notably, an optimal balance between immature precursor cells and more committed effector cells has previously also been found to be critical for regulating CD8<sup>+</sup> T cell immunity (53). In addition, a higher proportion of total immature memory CD8<sup>+</sup> and CD4<sup>+</sup> T cells are associated with improved prognosis in chronic HIV-1 infection (54) and can be enriched in the absence of viremia and immune activation in treated HIV-1-positive individuals (55). Interestingly, we found evidence that Notch signaling, previously mostly shown to regulate hematopoietic and thymic precursor cell differentiation (37, 38), is also involved in regulating the transition of long-lasting PD-1<sup>lo</sup> into short-lived PD-1<sup>hi</sup> Tfh-like cells. This suggests that stem cell pathways, previously recognized in the context of traditional organ-specific stem cells, may also contribute to regulation and fate decisions within the memory Tfh pools, and may offer molecular targets for selectively influencing memory Tfh development. A role for Notch in Tfh memory evolution is also consistent with the recent observation that Notch signaling was necessary for Tfh differentiation in experimental animal models (42).

Taken together, these studies demonstrate that the CXCR5<sup>+</sup>CXCR3<sup>+</sup>PD-1<sup>lo</sup> Tfh-like population serves as precursor cells for PD-1<sup>hi</sup> cells, supports B cell development through cytokine secretion, is associated with maintenance of more broadly neutralizing HIV-1 antibody responses in the absence of high-level HIV-1 viremia, and can be selectively primed by DCs from HIV-1 controllers with neutralizing antibody responses. These studies may help to delineate mechanisms for inducing HIV-1 antibodies with higher levels of neutralizing breadth by prophylactic vaccines.

## Methods

*Study participants.* HIV-1 controllers who had maintained <1,500 copies/ml HIV-1 viral loads (VL) for a median of 5 years (range 2–14) in the absence of antiretroviral therapy, with (neutralizers [NT],  $n = 25$ , median VL 123 copies/ml, range 20–1,400 copies/ml; median CD4 counts 769.5 cells/ml, range 418–1,545 cells/ml) or without (non-neutralizers [NN],  $n = 20$ ; median VL 75 copies/ml, range 48–1,470 copies/ml; median CD4 counts 844.5 cells/ml, range 407–2,117 cells/ml) neutralizing antibodies against

Tier-2/3 HIV-1 viruses; untreated chronic progressors (CP;  $n = 14$ , median VL 28,352.5 copies/ml, range 2,368–90,500 copies/ml; median CD4<sup>+</sup> T cell counts 313 cells/ml, range 88–955 cells/ml); ART-treated chronically HIV-1–infected patients with suppressed HIV-1 viremia (H;  $n = 14$ , median VL <49 copies/ml; median CD4<sup>+</sup> T cell counts 490 cells/ml, range 183–964 cells/ml); and HIV-1–seronegative healthy persons (NG;  $n = 14$ ) were recruited for this study. Samples of mononuclear cells extracted from inguinal lymph nodes were obtained by surgical excision from HIV-1–negative study persons. Mononuclear samples from human tonsil tissue of HIV-1–negative individuals who underwent routine tonsillectomies were obtained as previously described (52).

*Analysis of the neutralizing breadth of HIV-1–specific antibodies.* As previously described (56), HIV-1 neutralization breadth was measured in a TZM-bl cell–based pseudovirus neutralization assay against a panel of Env pseudoviruses derived from 9 clade B tier 2 and 2 tier 3 neutralization sensitivities: tier 2: AC10.0.29\*, RHPA4259.7\*, THRO4156.18\*, REJO4541.67\*, WITO4160.33\*, TRO.11\*, SC422661.8\*, QH0692.42\*, CAAN5342.A2#; and tier 3: PVO.4\* and TRJO4551.58\*. The clade B and C isolates are denoted by the superscripts \* and #, respectively. Neutralization was defined as at least 50% inhibition of infection at a 1:20 dilution. The neutralization breadth was defined as the percentage of the 11 isolates neutralized by each plasma sample. All samples were screened for non–HIV-1–specific neutralization using murine leukemia virus pseudotyped virions

*Cell isolation.* Total CD19<sup>−</sup> BDCA1<sup>+</sup> and BDCA3<sup>+</sup> mDCs were purified from total PBMCs or lymph node suspensions by immunomagnetic enrichment as previously described (47) (purity >90%). Naive CD4<sup>+</sup> T cells and naive CD27<sup>−</sup> B cells were isolated using immunomagnetic negative selection kits (Miltenyi Biotec, Naive CD4<sup>+</sup> T Cell Isolation Kit II and Naive B Cell Isolation Kit II, human), leading to cell purity of >95%. MS and LD columns and/or the AutoMACS (Miltenyi Biotec) system were used for cell isolation.

*In vitro DC-based priming of Tfh-like cells.*  $5 \times 10^4$  freshly isolated naive CD4<sup>+</sup> T cells were cocultured with  $5 \times 10^4$  autologous naive B cells in the presence or absence of  $5 \times 10^4$  allogeneic mDCs from different patient cohorts in 96 round-bottom-well plates for 7 days. Naive T and B cells were obtained from the same donors, but different individuals were used for each experiment. Presence of CXCR5<sup>+</sup>PD-1<sup>+</sup> Tfh-like cells was analyzed by flow cytometry at the end of the culture. Naive B cells and T cells cultured alone with media only were used as negative controls. On day 7 of culture, supernatants were collected for cytokine and antibody quantification, and cells were harvested to analyze the phenotype of cultured T and B cells. When indicated, cells were cultured in the presence of 5 μg/ml anti–human CD40 neutralizing mAb (clone 82102, R&D Systems) or an isotype control mAb (Invitrogen). In some experiments, T-B-mDC cocultures were conducted in the presence of either 100 nM GSI (MK-0752; Selleck Chemicals) or DMSO as a control. In vitro generation of Tfh-like cells under contact-dependent and -independent conditions was evaluated by coculturing naive B cells and T cells as previously described in the lower chamber of 96-well Transwell plates (Corning) directly in contact with mDCs or with mDCs plated on the upper Trans-well chamber for 7 days.

*Functional assays with ex vivo isolated human pTfh populations.* Human CXCR3<sup>+</sup> and CXCR3<sup>−</sup> PD-1<sup>lo</sup> and PD-1<sup>hi</sup> CXCR5<sup>+</sup> CD4 T cells (pTfh) were sorted in parallel with total CD3<sup>−</sup>CD19<sup>+</sup> B cells in PBMCs from healthy donors and cultured in the presence of 2 μg/ml SEB for 6 days. On day 6 of culture, supernatants were collected for cytokine and antibody quantification, and cells were harvested to analyze the phenotype of cultured pTfh and B cells. In some experiments, pTfh cells were labeled with 5 M CFSE (Life Technologies) to track cell proliferation in the indicated conditions.

*Luminex analysis.* Concentrations of human cytokines (IL-6, IL-10, IL-21, IL-12p70, IL-22, TNF-α, GM-CSF) and Igs (IgM, IgA, IgG) were analyzed in culture supernatants using Human Th17 magnetic bead and Human Immunoglobulin magnetic Luminex kits (Millipore), respectively, following the manufacturer's instructions.

*Gene expression analysis by RNA sequencing.* Total RNA was obtained from sorted CD14<sup>−</sup>CD11c<sup>hi</sup>HLADR<sup>+</sup> mDCs from peripheral blood of controller neutralizers ( $n = 4$ ) and controller non-neutralizers ( $n = 4$ ). Subsequently, total RNA was extracted using a commercial kit recommended for low numbers of cells (*mirVana Isolation Kit*, Life Technologies). RNA sequencing (RNA-Seq) libraries from mDCs from each patient group were generated as previously described (57). Briefly, whole transcriptome amplification (WTA) and tagmentation-based library preparation were performed using SMART-seq2 (57), followed by sequencing on a NextSeq 500 instrument (Illumina). FASTQ files were obtained using the Illumina demultiplexing pipeline. STAR (58) and RSEM (59) was used to align to

HG38 and to calculate the raw counts for genes, respectively. Transcripts per million (TPM) values were then normalized among all samples using the upper quantile normalization method. Subsequently, genes differentially expressed between mDCs from controller neutralizers and non-neutralizers were identified by DESeq2 (30). Ingenuity Pathway Analysis (IPA; QIAGEN) was used to functionally categorize differentially expressed genes and to biocomputationally identify putative upstream regulators responsible for differential gene expression signatures. The original RNA-seq data are available in the NCBI's Gene Expression Omnibus (GSE90897).

**Flow cytometry.** Ex vivo and cultured PBMCs, as well as tonsil and lymph node cell extracts, were stained with LIVE/DEAD cell blue viability dye (Invitrogen) and different panels of mAbs. For Tfh/pTfh identification or characterization, the following mAbs were used: anti-human CD4 (RPA-T4, BD), CD3 (OKT3), CXCR5 (J252D4), CXCR3 (G025H7), PD-1 (H12.2H7), ICOS (C.398.4A) (BioLegend). For phenotypical analysis of cultured B cells, anti-CD19, -CD38 (BioLegend), -CD27 (M-T271, BD) mAbs were included in the panel. In addition, mDCs from different study cohorts were characterized using anti-human PD-L1 (29E-2A3), CD40 (5C3), CD86 (IT 2.2), HLA-DR (L243), CD11c (clone 3.9), ICOS-L (2D3) (BioLegend), and CD14 (MøP9, BD). mDCs were identified from bulk PBMCs as a population of viable CD14<sup>-</sup> lymphocytes expressing high levels of CD11c and HLA-DR. For intracellular staining, cells were treated with a commercial fixation/permeabilization kit (BioLegend) according to the manufacturer's protocol and incubated with anti-human Bcl-6 (K112-91, BD) and Tbet (4B10, BioLegend) mAbs. Subsequently, samples were analyzed on a Fortessa cytometer (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).

**Statistics.** Significance of phenotypic differences between the different patient cohorts was assessed using Mann Whitney *U* tests for individual comparisons or Kruskal-Wallis test followed by Dunn's test for multiple comparisons. To calculate significance of differences observed within the same individuals or experiments with different treatments, a Wilcoxon matched-pairs signed-rank test or a Friedman test followed by a Dunn's tests was used for individual or multiple comparisons, respectively. To analyze statistically significant differences in HLA-I and HLA-II allele distribution between controller neutralizers and non-neutralizers, a  $\chi^2$  test was used, and *P* values were adjusted by the Bonferroni correction. To investigate transcriptional expression patterns in mDCs from neutralizers and non-neutralizers by RNA-Seq, DESeq2 (30) was used to access the differentially expressed genes after using STAR (58) and RSEM (59) for the alignment to HG38. Data were visualized as heatmaps, with unsupervised hierarchical clustering analysis using the Ward method. In addition, the significant canonical pathways and upstream regulators were predicted by IPA using Fisher's exact test. *P* values less than 0.05 were considered significant.

**Study approval.** All subjects gave written informed consent, and the study was approved by the Institutional Review Board of Massachusetts General Hospital/Partners Healthcare. Individuals undergoing lymph node biopsy procedure were recruited at the University of Hamburg (Germany) and at Massachusetts General Hospital in Boston, according to protocols approved by the Institutional Review Board of Partners Healthcare and University of Hamburg. Human tonsil samples were obtained at Martin Memorial Hospital, Florida, following a protocol approved by the hospital's IRB.

## Author contributions

XGY, EMG, and M Lichtenfeld developed the research idea and study concept, designed the study, and wrote the manuscript. XGY supervised the study. EMG designed and conducted most experiments. JC and TH provided technical help in most experiments. ZO supervised statistical analysis and provided bioinformatic support for RNA-Seq data analyses. M Lindqvist and DEK provided some phenotyping data on lymph node samples from HIV-1-negative donors. AKS and KEK performed the RNA-Seq experiments. BDW provided PBMC samples from HIV-1-infected patients. JSZW, JVL, M Lindqvist, FP, and DEK provided human lymph node samples. RC and EKH provided human tonsil samples. BDW, RC, AKS, DEK, and EKH participated in study-related discussions and critically reviewed the manuscript.

## Acknowledgments

This work was supported by the NIH (grants AI078799, AI089339, HL121890, AI098484, HL126554, AI116228 and AI087452 to XGY; AI098487 and AI106468 to M Lichtenfeld). PBMC sample collection was supported by the Bill and Melinda Gates Foundation (OPP 1066973), the Mark and Lisa Schwartz Foundation, the Ragon Institute of MGH, MIT and Harvard, and the International HIV Controller

Consortium. AKS was supported by the Searle Scholars Foundation, the Beckman Young Investigator Program, and an NIH New Innovator Award (DP2 OD020839). DEK is supported by a Research Scholar Career Award of the Quebec Health Research Fund (FRQS). Lymph node sample collection was supported by NIH Scripps CHAVI-ID (1UM1-AI100663 to BDW and DEK); tonsil sample collection was supported by NIH grant AI106482 to EKH.

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