



Published in final edited form as:

Arthritis Rheumatol. 2015 April ; 67(4): 988–999. doi:10.1002/art.39020.

Circulating Follicular Helper-Like T Cells in Systemic Lupus Erythematosus: Association with Disease Activity

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Abstract

Objective—To assess circulating follicular helper-like CD4⁺ T (cTfh-like) cells in systemic lupus erythematosus (SLE) and determine their relationship to disease activity.

Methods—We analyzed blood samples from SLE patients, and as controls, Behçet's disease (BD) patients and healthy individuals. We used flow cytometry to enumerate cTfh-like cells using as markers the C-X-C chemokine receptor type 5 (CXCR5), inducible T-cell costimulator (ICOS), programmed cell death protein-1 (PCDC1, PD-1), and secretion of interleukin-21 (IL-21). We compared the frequency of cTfh-like cells with that of circulating plasmablasts (CD19⁺IgD⁻CD38⁺) and evaluated their possible association with disease activity.

Results—cTfh-like T cells, identified as CXCR5^{hi}ICOS^{hi}PD-1^{hi}, were expanded in the blood of SLE patients compared to BD and healthy controls. Such cells produced IL-21 with lower expression of CCR7, compared to circulating CXCR5^{hi} central memory (T_{cm}) cells, enabling their distinction. PD-1, not ICOS or CXCR5, expression was significantly elevated in cTfh-like cells from SLE patients compared to controls. PD-1 expression among CXCR5^{hi} cTfh-like cells correlated with disease activity, circulating plasmablasts, and anti-dsDNA antibody positivity, but not disease duration nor past organ injury; rather, it reflected current active disease.

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The authors have no financial conflicts of interest to disclose regarding this work.

Conclusion—We found that cTfh-like cells are associated with disease activity in SLE, suggesting that their presence indicates abnormal homeostasis of T-B cell collaboration with a causal relationship central to disease pathogenesis. These findings also suggest that cTfh-like cells provide a surrogate for aberrant GC activity in SLE, and that their PD-1 expression offers a tool for following disease activity and response to therapies.

Systemic lupus erythematosus (SLE, lupus) is marked by immune complex-mediated tissue injury in multiple organs. The clinical manifestations and the immunoregulatory factors that contribute to disease are diverse. Identification of common pathogenic pathways and the corresponding biomarkers that link abnormal cellular activity to disease activity are necessary to define therapeutic targets.

Central to antibody production is the collaboration between CD4⁺ T cells and B cells in germinal centers (GC) of secondary lymphoid organs (SLOs), the site of immunoglobulin (Ig) isotype switching and affinity maturation, with the subsequent genesis of memory B cells and long-lived plasma cells (PCs) (reviewed in (1, 2)). Pathogenic autoantibodies in murine and human lupus are also class-switched and somatically mutated with affinity maturation (3, 4), and arise from autoreactive memory B cells upon restimulation (5-7), features consistent with GC selection. The role of aberrant GC responses in the autoantibody genesis finds support in the observation that spontaneous GCs form in murine lupus (8), with evidence of exuberant GC activity in patients with active lupus nephritis (9). These data indicate that autoreactive B-cell maturation occurs in GCs in SLE.

Follicular B-helper T (Tfh) cells are necessary for T cell-dependent B-cell maturation in the GC (reviewed in (1, 2)). Tfh cells express the transcription factor B-cell lymphoma 6 (Bcl6) that drives a gene program critical for their development and function (10-12). Tfh cells are identified by a combination of markers, including CXCR5 (C-X-C chemokine receptor type 5) that enables their migration along a CXCL13 (C-X-C motif chemokine 13) gradient into B-cell follicles with subsequent GC formation (13, 14); ICOS (inducible T-cell costimulator), necessary for development of nascent Tfh cells upon their activation by dendritic cells (DCs) expressing ICOS ligand (ICOS-L) (15), and for their subsequent expansion upon interactions with ICOS-L expressed on B cells (16, 17); and PD-1 (programmed cell death protein-1; also PCDC1), which provides inhibitory signals to T cells (18), but also regulates GC B-cell selection and survival necessary for formation of long-lived PCs (19) of the type observed in SLE (4, 7). Tfh cells secrete interleukin (IL)-21, critical for GC development and maintenance (20, 21), and for Ig class switching and PC development (22). Aberrant expansion of Tfh cells is causally linked to abundant GCs, autoantibodies, and end-organ damage in murine lupus (23-25). Phenotypically similar T cells (20, 24) drive autoreactive B-cell responses occurring outside of GCs in murine SLOs (26) and in the kidneys of SLE patients (27). Thus, Tfh cells are central to disease in mice and humans.

Although human Tfh cells can be analyzed in spleens and tonsils, their assessment in SLE has been hampered by the inability to routinely sample SLOs. However, cells with a similar CXCR5^{hi}PD-1^{hi} phenotype circulate, potentially providing a window into analysis of Tfh cells in SLOs. For example, a PD-1^{hi} subset of CD4⁺ CXCR5^{hi} T cells expands transiently

following influenza immunization, in conjunction with influenza-specific antibody-secreting cells (28, 29). HIV-infected individuals with neutralizing HIV-specific antibodies also have increased numbers of circulating CD4⁺ CXCR5^{hi} PD-1^{hi} T cells (30). CXCR5^{hi}PD-1^{hi} cells, some of which are ICOS^{hi}, circulate in SLE patients (29, 31) and in patients with juvenile dermatomyositis. Circulating CD4⁺ CXCR5^{hi} T cells from non-autoimmune individuals drive *in vitro* differentiation of naïve (30) and memory B cells (28, 30) into Ig-secreting PCs, analogous to tonsillar Tfh cells, findings supporting their functional capability. While the circulating CD4⁺ CXCR5^{hi} pool does not express the canonical Tfh-cell transcription factor Bcl6 (28-32), these studies nonetheless suggest that blood-borne CD4⁺ CXCR5^{hi} cells represent a Tfh-cell counterpart and one that reflects the GC response (reviewed in (2)).

Characterization of circulating CD4⁺ CXCR5^{hi} T cells is confounded by the presence of phenotypically similar blood-borne central memory (Tcm) CD4⁺ T cells, a portion of which express CXCR5. Yet, Tcm cells can be distinguished from the circulating CD4⁺CXCR5^{hi}PD-1^{hi} T cells that that expand in autoimmunity and transiently following vaccination as they are PD-1^{lo} and ICOS^{lo} (33). They also express the C-C chemokine receptor type 7 (CCR7) and L-selectin (CD62L) needed by these circulating cells for re-entry into lymph nodes where they become available for antigen rechallenge (14, 34), and differentiation into effector T helper (Th) cell subsets, among them Tfh cells (29, 35, 36). Tcm cells upon re-activation are presumably competent to re-enter B-cell follicles (29) via expression of the CXCR5 receptor interacting with its follicular ligand CXCL13, and to drive Ig secretion by naive and memory B cells, promoting secondary humoral immunity (32, 33).

Patients with SLE have expansion of circulating CD4⁺ CXCR5^{hi} PD-1^{hi} cells at constant levels that is correlated with autoantibody titers (29, 31). Yet, the relationship of circulating CD4⁺ CXCR5^{hi} subsets to disease activity and the aberrant B-cell response is uncertain, partly due to the challenge discriminating among CXCR5^{hi} Tfh-like and Tcm populations. Thus, we sought circulating Tfh-like (cTfh-like) cells in lupus patients, correlating them with clinical history, disease activity, and pathological B-cell function. We found that the frequency of cTfh-like cells was increased compared to controls, with expression of PD-1 most strongly associated with disease activity, suggesting the capacity for B-cell help. cTfh-like cells could be distinguished from Tcm cells via PD-1 upregulation and CCR7 downregulation, and IL-21 competence with evidence of recent cell division, suggesting functional capability. These findings suggest that cTfh-like cells provide a surrogate for aberrant GC activity in SLE, and their PD-1 expression offers a tool for following disease activity and therapeutic responsiveness.

PATIENTS AND METHODS

Study populations

We analyzed blood samples from two adult cohorts. The first, from the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, included 49 SLE patients (46 female and 3 male; median age 37 years, range 30-48 years; 32 Caucasian, 17 non-Caucasian), 28 Behçet's disease (BD) patients (22 female and 6 male; median age 48 years, range 40-56 years; 20 Caucasian, 8 non-Caucasian), and 16 healthy controls (12 female and

4 male; median age 28 years, range 27-32 years; 13 Caucasian, 3 non-Caucasian). Their characteristics are shown in Supplementary Table 1, with phenotypic data summarized in Table 1 and Figures 1-4. We also studied samples from 8 patients with SLE (8 females; median age 40.38 years, range 26 and 60 years; 3 Caucasian, 5 non-Caucasian) from Yale-New Haven Hospital (YNHH), with their data shown in Figure 5. SLE patients fulfilled revised disease criteria of the American College of Rheumatology (ACR) with disease activity assessed by the SLE Disease Activity Index (37), whereas BD patients met criteria set by the International Study Group for Behcet's Disease (38, 39). Informed consent was obtained from all subjects. This study was approved by the institutional review committees of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo and Yale University.

Flow cytometry

We isolated peripheral blood mononuclear cells (PBMC) using density-gradient centrifugation on Ficoll-Paque with single cell suspensions stained with the following antibodies: Alexa Fluor 700-conjugated CD4, V450-conjugated CD45RA and CCR7, PE-conjugated CD4 and CCR6, PE-Cy7-conjugated PD-1, CXCR3, and IgD, Alexa Fluor 647-conjugated CXCR5 and IL-21, fluorescein isothiocyanate (FITC)-conjugated CD62L, APC-Cy7-conjugated CD19, APC-H7-conjugated CD3, PE-Cy5-conjugated CD38, streptavidin-conjugated APC-Cy7 (all from BD PharMingen), and FITC-conjugated ICOS, PE-conjugated Bcl6, and PE-Cy5 conjugated TCR β (from eBioscience), and PE-conjugated Ki-67 (from BioLegend). Stained cells were analyzed by multiparameter flow cytometry (LSRFortessa or LSR II, BD), with exclusion of doublets by forward and side scatter, using FlowJo software (Tree Star).

Intracellular IL-21, Bcl6, and Ki-67 staining

Surface-stained PBMC were sorted by flow cytometry (FACS Aria, BD Biosciences) into naïve (CD45RA⁺), cTfh-like (CD45RA⁻CXCR5^{hi} ICOS^{hi}PD-1^{hi}), and Tcm (CD45RA⁻CXCR5^{hi}ICOS^{lo}PD-1^{lo}) populations, with the latter distinguished by CCR7 expression (34). Sorted subsets were stimulated with PMA (50 ng/ml) and ionomycin (1 μ g/ml) for 2 hours with Golgi Plug (BD) for an additional 2 hours. Cells were fixed (BD CytoFix/CytoPermTM) and permeabilized (BD PERM/WashTM), and stained with anti-IL-21. For Bcl6 and Ki-67 staining, surface-stained PBMC were fixed and permeabilized without stimulation.

Autoantibody profile and serum complement levels

Antinuclear and anti-double-stranded DNA (anti-dsDNA) antibodies were detected by indirect immunofluorescence (IIF) using as substrates HEp-2 cells or *Crithidia luciliae*, respectively (INOVA Diagnostics Inc). Serum levels of anti-Ro(SS-A), anti-La(SS-B), anti-RNP, and anti-Sm antibodies were determined by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (INOVA Diagnostics Inc). Total hemolytic complement activity (CH100) was measured by the immunohemolysis assay (normal range 150-350 U/ml).

Statistical analysis

All data are presented as the mean \pm SEM. The significance of the difference between groups was analyzed with one-way ANOVA test, with the significance of difference between two groups evaluated by the two-tailed Student's *t*-test. Spearman correlation coefficient or Pearson correlation coefficient with two-tailed *p* value were determined in the analysis of correlations. *P* values less than 0.05 were considered significant. Data were analyzed with Prism software (version 5.0d for Macintosh; GraphPad Software).

RESULTS

Analysis of cells with a Tfh phenotype in the blood of SLE patients and controls

CXCR5^{hi} Tfh cells in SLOs express ICOS and PD-1, central to their development and helper function in B-cell follicles and GCs (reviewed in (2)). To identify cells with a Tfh-cell phenotype in the blood of lupus patients and controls, we sought CD4⁺ T cells that were CXCR5^{hi}ICOS^{hi}PD-1^{hi}, markers that distinguish them from CD4⁺ CXCR5^{hi}CCR7^{hi}ICOS^{lo}PD-1^{lo} Tcm cells observed in the circulation of normal individuals.

We analyzed single CD4⁺ TCR β ⁺ cells, dividing them into naïve CD45RA⁺ and activated CD45RA⁻ subsets, with the CD45RA⁻ subset further divided as CXCR5^{hi} or CXCR5^{lo} (Figure 1A, left panel) followed by gating on PD-1 and ICOS (Figure 1B). Flow cytometry gates were set according to the basal expression of PD-1 and ICOS (both < 0.5% positive), or that of the subsequent ICOS^{hi}PD-1^{hi} double positive population (< 0.1% positive), on naïve CD45RA⁺ CD4⁺ T cells from healthy controls (Figure 1A, right panel). Naïve CD45RA⁺ T cells, regardless of donor's health status, were primarily CXCR5^{lo} and largely lacking ICOS and PD-1 expression (Figure 1A, right panel; representative sample from a healthy control). We found only a few circulating activated CD45RA⁻CXCR5^{hi}ICOS^{hi}PD-1^{hi} cells, *i.e.*, those bearing a Tfh-cell phenotype, in healthy controls (Figure 1B, left panel and Figure 1C), consistent with previous reports (28-32); CD45RA⁻CXCR5^{lo} cells from normal donors were also largely PD-1^{lo}ICOS^{lo} (Figure 1C). By contrast, CD45RA⁻CXCR5^{hi}ICOS^{hi}PD-1^{hi} cells were expanded in the blood of lupus patients (Figure 1B, middle panel, representative example, and Figure 1C), with correlation of PD-1 and ICOS expression (Figure 1E, left panel). Expression of both on tonsillar Tfh cells was higher than on circulating CXCR5^{hi}ICOS^{hi}PD-1^{hi} cells from SLE patients: the mean fluorescence intensity (MFI) \pm standard deviation (SD) of PD-1 and ICOS was 10000 \pm 1587 and 12210 \pm 1481, respectively, on the former (*n* = 3), compared to an MFI \pm SD of PD-1 and ICOS of 4290 \pm 543 and 5827 \pm 1583, respectively, on the latter cells (*n* = 21) (Figure 1B, representative examples). tonsillar Tfh cells also expressed Bcl6 (40), the canonical Tfh-cell transcription factor, whereas the circulating CXCR5^{hi} population did not (28-32) (Figure 1D). CXCR5^{hi}ICOS^{hi}PD-1^{hi} cells were not expanded in blood of patients with BD (Figure 1C), indicating that their presence is not a function of this inflammatory disorder. We noted an increase in the percentage of ICOS^{hi}PD-1^{hi} cells within the CXCR5^{lo} subset in lupus patients (Figure 1C), as compared to BD patients and healthy controls; however, PD-1 and ICOS expression was not correlated in this population (Figure 1E, right panel), unlike in the CXCR5^{hi} subset (Figure 1E, left panel). Thus, circulating CD4⁺CD45RA⁻CXCR5^{hi} cells in lupus contained a population resembling Tfh cells found

in SLOs, albeit with lower PD-1 and ICOS expression, and without Bcl6; we considered these cTfh-like cells.

We next asked if individual Tfh-cell markers CXCR5, ICOS, and PD-1 were selectively upregulated on circulating CD4⁺TCRβ⁺ cells in lupus. Patients with active disease have increased circulating CD4⁺ICOS^{hi} lymphocytes, although their number varies according to definition of this population and the cohort (41). We did not find that increased percentages of CXCR5^{hi} or CXCR5^{lo} cells *per se* were associated with SLE (Figure 2A, 2B), or that ICOS expression, as measured by MFI, distinguished circulating CD4⁺ T cells from patients and controls (Figure 2C). By contrast, expression of PD-1, a molecule integral to GC maturation and PC genesis (19), was increased in the CXCR5^{hi} subset in lupus, compared to those from healthy controls and BD patients (Figure 2D). The increase in PD-1 MFI appeared to account for the increase in CXCR5^{hi} ICOS^{hi} PD-1^{hi} cTfh-like cells we observed in the lupus patients (Figure 1C). PD-1 also was upregulated in the CXCR5^{lo} subset in SLE, but less so than in CXCR5^{hi} cells. These data demonstrate that CXCR5^{hi}ICOS^{hi}PD-1^{hi} cTfh-like cells are expanded in SLE, and PD-1, but not ICOS, is selectively up-regulated on the circulating CXCR5^{hi} subset.

Correlation between cTfh-like cells and disease activity in SLE

We next asked if the expansion of cTfh-like cells in lupus patients was correlated with disease activity, as measured by SLEDAI. We did not find a significant relationship between the percentage of the CXCR5^{hi}ICOS^{hi}PD-1^{hi} subset and this scale (Spearman's $r = 0.26$, $p = 0.19$; data not shown), and we did not observe a correlation between the former and organ involvement and aberrant B-cell activation (data not shown). Yet, as the cTfh-like markers CXCR5, ICOS, and PD-1 are differentially regulated in cells from patients (Figure 2), we asked if their individual expression correlated with clinical parameters. We found a correlation between the SLEDAI and the MFI of PD-1 (Figure 3A), but not with ICOS MFI and CXCR5^{hi} cells (Figures 3B and C, respectively). We observed a similar correlation between PD-1 expression on the CXCR5^{lo} subset and disease activity (Figure 3D), suggesting possible Th17 and/ or Th1-cell involvement in SLE (42), as these Th subsets are CXCR5^{lo}. Although the elevated PD-1 MFI could reflect T cell activation common in active SLE (41), this did not appear to account for the relationship between it and the SLEDAI, as the latter was not correlated with expression of ICOS, also a marker of activated T cells. Moreover, the percentage of activated CD45RA⁻ CD4 T cells was not related to the SLEDAI (data not shown).

Given the correlation between PD-1 expression on CXCR5^{hi} cells and disease activity, we asked if the former were linked to organ involvement (Table 1). In patients with proteinuria and hematuria, compared to those without, we found an increase in PD-1 MFI on CXCR5^{hi} cells (1911 vs. 1199, respectively; $p = 0.02$), as we did for patients with ongoing pyuria (2590 vs. 1200; $p = 0.0007$). By contrast, apart from serositis (1551 vs. 1087, $p = 0.007$), PD-1 expression was not correlated with organ injury, including in the skin, joints, kidney, CNS, and vasculature. These results suggested that the PD-1 MFI on CXCR5^{hi} cells correlated with disease activity, including renal inflammation. Consistent with these findings, we found that PD-1 MFI on the CD4⁺ CXCR5^{hi} subset was increased in patients

with elevated anti-dsDNA antibodies, compared to patients without such autoantibodies (1891 vs. 1169, respectively; $p = 0.004$), and was likewise elevated in hypocomplementemic patients (1862 vs. 1173, $p = 0.006$) as measured by total hemolytic complement activity, with the latter presumably reflecting consumption upon engagement by autoantibody-autoantigen complexes.

Association between PD-1 expression and the expansion of circulating plasmablasts

Considering the pivotal role of PD-1 expressed on Tfh cells in GC maturation and PC genesis (19) and the positive association between PD-1 expression on blood CXCR5^{hi} CD4 cells and anti-dsDNA antibodies with presumed complement consumption, we asked if the elevated expression of PD-1 was correlated with B-cell activation. We purified lymphocytes from patients and controls, staining them with CD19, IgD, and CD38 to assess the pool of the IgD⁻CD38^{hi} plasmablasts (9). Consistent with previous reports (9, 43), we found an increased percentage of circulating plasmablasts in patients compared to healthy controls (Figure 3E), with their percentage similar regardless of treatment (Figure 3F). We also found that the PD-1 MFI on CXCR5^{hi}, and on CXCR5^{lo}, cells correlated with the plasmablast percentages among circulating B cells (Figure 3G and H, respectively); removal of outliers had no effect on statistical analyses.

Analysis of CXCR5^{hi} Th-cell subsets and correlation with SLEDAI

Differential expression of the chemokine receptors CXCR3 and CCR6 can be used to separate CXCR5^{hi} cells into effector subsets expressing cytokines characteristic of Th1, Th2, and Th17 cells, with production of IFN- γ , IL-4, and IL-17, respectively (32, 44). Patients with SLE have among the CXCR5^{hi} CD4⁺ population increased frequencies of IL-17-producing CCR6⁺CXCR3⁻ and IL-4-producing CCR6⁻CXCR3⁻ subsets, with a decrease in IFN γ -secreting CCR6⁻CXCR3⁺ cells (32, 40, 44). In our hands, these CXCR5^{hi} subsets all produced IL-21, while individually producing IL-4, IFN- γ , and IL-17, respectively, in accord with chemokine receptor expression (data not shown). Although we did not observe differences in their relative frequencies in our cohort (Figure 4A-D), we found that the frequency of the CCR6⁻CXCR3⁻ subset was associated with disease activity, replicating earlier findings (32, 44), while that of the CCR6⁺CXCR3⁺ subset was negatively associated (Figure 4E-F).

Relationship of CXCR5^{hi} cTfh-like and CXCR5^{hi} Tcm cells in SLE

CXCR5^{hi} cells in the circulation of healthy individuals display a Tcm-cell phenotype: they are ICOS^{lo}PD-1^{lo} with elevated expression of CCR7 and CD62L (14, 34), required for interaction with CCL19 and 21, chemokines abundantly expressed by high endothelial venules and T zone reticular cells, and with vascular addressins, respectively, with CXCR5 aiding follicular homing for B-cell helper function (14). We used these markers to distinguish CXCR5^{hi} cTfh-like and CXCR5^{hi} Tcm cells in the circulation of lupus patients. To establish staining parameters, we analyzed CD4⁺TCR β ⁺CD45RA⁺ naïve cells, which also use CCR7 to home to SLOs, showing they robustly expressed CCR7 and were PD-1^{lo} (Figure 5A, left panel). Activated CD45RA⁻CXCR5^{hi} cells could be divided into PD-1^{lo} CCR7^{hi} and PD-1^{hi} CCR7^{lo} populations (Figure 5A, right panel), although the former,

defined as Tcm cells (29, 34), expressed less CCR7 than did naïve CD4⁺TCRβ⁺ cells (Figure 5B, left panel). CXCR5^{hi}PD-1^{hi} cells expressed less CCR7 than did Tcm cells (Figure 5B). A greater percentage of these cells, compared to the Tcm population, also stained for the proliferation marker Ki-67 (Figure 5C), consistent with a proliferation history of cTfh-like cells in contrast to quiescent Tcm cells. Thus, PD-1 expression, with that of CCR7, is a marker for distinguishing cTfh-like from Tcm cells (29).

We next stained for intracellular IL-21, a cytokine critical for the B-cell helper function of Tfh cells, with the idea that cTfh-like cells would have cytokine production upon stimulation. While few if any naïve CD4 cells produced IL-21, activated CXCR5^{hi}PD-1^{hi} cells from the lupus patients expressed this cytokine, producing more in comparison to CXCR5^{hi}PD-1^{lo} Tcm cells (Figure 5D) as expected, since non-Tfh central memory cells, such as those bearing a Th1 phenotype, produce less cytokines than their effector counterparts (34). These data suggest that cTfh-like cells have functional capacity similar to that of Tfh cells found in SLOs, with PD-1 and IL-21 expression.

Discussion

Expansion of Tfh cells is associated with the development of systemic autoimmunity in murine lupus (23, 24), with linkage to heightened GC responses and end-organ damage (25). Patients with SLE also have altered GC homeostasis (4, 7, 9, 45), suggesting that Tfh cells are likewise aberrantly regulated in the human disease. With these ideas in mind, we asked if cells bearing a Tfh-like phenotype circulated in SLE, and if so, did their presence reflect aberrant GC homeostasis.

We found an increase in the number of cTfh-like cells in the blood of lupus patients, compared to controls. In addition to upregulation of PD-1 and ICOS, cTfh-like cells in SLE down-regulated CCR7, a Tcm-cell marker (34), with this combination enabling distinction between these two populations. Expansion of cTfh-like cells was not seen in Behçet's disease, suggesting that it was not a function of a systemic inflammatory disorder, but a distinctive phenotype in SLE. The cTfh-like cells included those recently proliferating, as determined by Ki-67 positivity, and that secreted IL-21, distinguishing them from functionally "exhausted" CD4⁺PD-1^{hi} cells found in patients with chronic infections or cancer (46, 47). In particular, the expansion of PD-1^{hi} CXCR5^{hi} cells reflected GC dysfunction in SLE, as evidenced by their correlation with circulating plasmablasts and autoantibodies.

Expansion of circulating IL-21-producing CD4⁺CXCR5⁺ T cells has been reported in SLE patients, with such cells expressing the Tfh-cell transcription factor Bcl6 (48). The latter finding is at odds with ours, and those of others, in which circulating cells lack Bcl6 (28-32), differences that perhaps reflect cell isolation or staining, or degree of GC dysfunction. Regardless, these findings indicate that Tfh-like cells circulate in SLE.

While we noted an increase in the frequency of cTfh-like cells in SLE compared to controls, this was not correlated with disease activity measured by SLEDAI. One factor contributing to this lack of correlation was the use of ICOS to identify cTfh-like cells. ICOS is

upregulated on all activated CD4⁺ T cells, and in our hands, its expression was nearly indistinguishable among patient and control groups (Figure 2C), negating its specificity for cTfh-like cell expansion. By contrast, PD-1 expression on CXCR5^{hi} cells was correlated with the SLEDAI, perhaps reflecting the aberrant GC reaction in SLE, as evidenced by a correlation with expansion of circulating plasmablasts and anti-ds-DNA antibodies with hypocomplementemia. Thus, the degree of PD-1 expression on cTfh-like cells, more so than their frequency, is an indicator of pathological B-cell responses and disease activity. It will nonetheless be important to validate the utility of PD-1 expression on blood CXCR5^{hi} cells as a biomarker for disease activity via longitudinal studies.

We also found that PD-1 expression on CD4⁺ CXCR5^{lo} cells, as for their CXCR5^{hi} counterparts, correlated with disease activity in SLE, as well as with circulating plasmablasts (Figure 3H). These associations may be secondary to the proinflammatory nature of activated CXCR5^{lo}PD-1^{hi} cells (42). Consistent with this idea are the observations that such cells upon activation upregulate CXCR5 with B-cell helper function (13), inducing memory B cells to produce class-switched Igs (32).

One contributing factor to elevated levels of PD-1 may be type I interferons (IFNs), as these correlate with disease activity (49, 50). Type I IFNs enhance humoral immunity and promote isotype switching *in vivo* (51), which in T-cell dependent responses, rely upon Tfh cells, although our recent murine data suggest that effects of these cytokines on Tfh-cell development need dissection in the context of signaling by other cytokines (52). Nonetheless, the heightened PD-1 expression on CXCR5^{hi} cells in SLE is consistent with its induction and maintenance by IFN- α in murine CD4⁺ T cells (53). We also found that IFN- α enhanced the expression of PD-1 on human Tfh cells (data not shown). Thus, we need in the future to dissect Tfh-cell differentiation and function in a type I IFN-rich environment, common to that in SLE (50, 54). Moreover, since polymorphisms of *PDCDI*, the gene encoding PD-1, are associated with SLE susceptibility in certain ethnicities (55, 56), understanding of PD-1-pathway regulation in Tfh and cTfh-like cells in SLE will require dissection of genetic and environmental precipitants.

PD-1 provides inhibitory signals to activated T cells, consequently helping maintain peripheral tolerance (18). Yet, engagement of Tfh-cell PD-1 via its ligands on GC B cells is required for appropriate production of T-cell cytokines critical for GC maturation and PC formation, with the absence of PD-1 signaling leading to Tfh cells that are less competent to provide B-cell help (19). Depletion of PD-1 bearing T cells also has a beneficial effect in murine lupus via reduction in autoantibody formation, cytokine production, and renal disease (57). On the other hand, blockade of the PD-1 ligand PD-L1 in murine lupus enhances proinflammatory cytokine and anti-dsDNA antibody production (57). These contrasting observations highlight the fine orchestration of the GC reaction in which PD-1 regulation of Tfh-cell function is necessary for provision of proper B-cell maturation signals at the expense of robust T-cell inflammatory function (19).

Why are cTfh-like cells found in the blood in SLE? They are likely distinct from Tfh cells. They lack expression of the canonical Tfh-cell transcription factor Bcl6 (28-32), critical for induction of the Tfh-cell program of differentiation (10-12). They are also found in mice and

humans with genetic deficiency in SAP, a SLAM (signaling lymphocyte activation molecule)-associated protein, essential for Tfh-cell differentiation and GC formation (29). These findings suggest that cTfh-like cells form, and are released from, SLOs prior to Tfh-cell formation and immigration into the GC. These cells also expand within a few days following flu vaccination, indicative of recall from the T_{cm} cells (28, 30), as well as arise from naïve cells following immunization of mice (29). They are functional with ability to promote Ig secretion by B cells, and may re-enter GCs to promote B-cell maturation. Thus, it seems likely that they arise from autoreactive naïve precursor and/or memory T cells in SLE, with the capacity to drive pathogenic autoantibody formation, as indicated by the data from others (29) and revealed herein.

In conclusion, we have shown that cTfh-like cells are expanded in the blood of patients with SLE, with linkage to disease activity, suggesting that they play a central role in pathogenesis. These data suggest that PD-1 expression on circulating CD4⁺ CXCR5^{hi} T cells may be a marker for GC B-cell dysregulation. It also may be useful for monitoring humoral responses in the context of disease activity and therapeutic responsiveness, while providing a basis to better elucidate the dynamics of pathological Tfh-cell activity in human SLE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported in part by FAPESP 2011/02119-0 (to EB), and by grants provided by AbbVie, the Alliance for Lupus Research and the NIH (AR040072 and AR053495) (to JC). SangTaek Kim was supported by a Scientist Development Award from the American College of Rheumatology Research Foundation and by NIH T32 AR07107.

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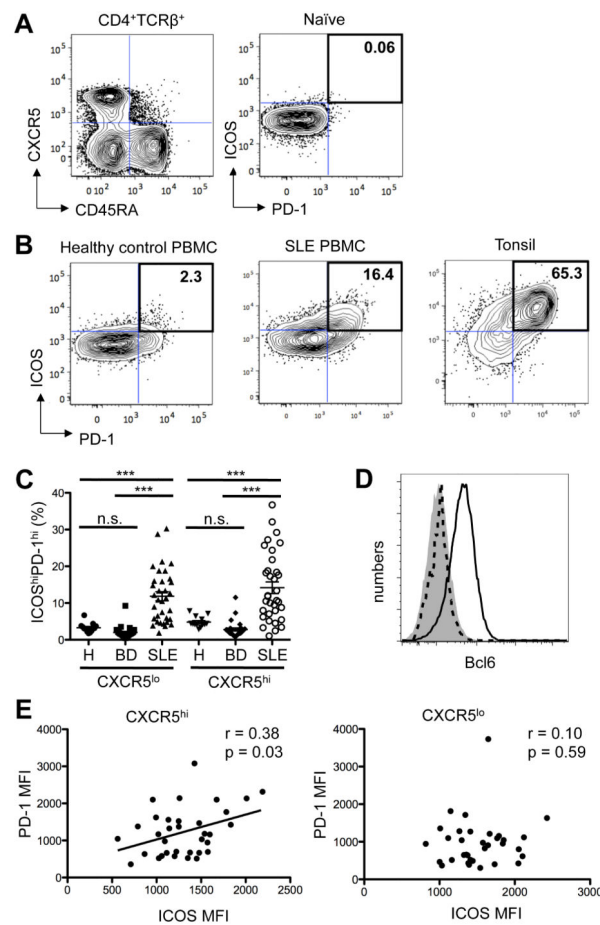


Figure 1.

Circulating CXCR5^{hi}ICOS^{hi}PD-1^{hi} Tfh-like cells are expanded in the blood of patients with SLE compared to healthy controls and patients with Behçet's disease. **A**, CD4⁺TCRβ⁺ T cells were separated into naïve (CD45RA⁺) and activated (CD45RA⁻) pools, with the latter subdivided into CXCR5^{hi} and CXCR5^{lo} subsets (left panel). Gates for the expression of PD-1 and ICOS were set using naïve CD4⁺ T cells from healthy controls (right panel). **B**, Contour plots of ICOS and PD-1 expression on CD45RA⁻ CXCR5^{hi} cells from a representative healthy control (left panel) and an SLE patient (middle panel), compared to tonsillar cells from a non-SLE donor (right panel). Values are the percentage of ICOS^{hi}PD-1^{hi} cells among CD4⁺ CXCR5^{hi} cells. **C**, Percentage of ICOS^{hi}PD-1^{hi} lymphocytes among CXCR5^{hi} and CXCR5^{lo} CD4⁺ T cells in patients with SLE ($n = 49$), Behçet's disease (BD; $n = 28$) and healthy controls (H; $n = 16$) (One-way ANOVA $***p < 0.001$). **D**, Staining of Bcl6 in naïve (shaded) and Tfh cells (black line) from a tonsillar sample, compared to circulating CXCR5^{hi}PD-1^{hi} cells (dotted line) from a representative SLE patient. **E**, Correlation between the MFI of PD-1 and ICOS on CXCR5^{hi} and CXCR5^{lo} CD4⁺ T cells (Pearson $r = 0.38$, $p = 0.03$, left panel, and Pearson $r = 0.10$, $p = 0.59$, right panel, respectively).

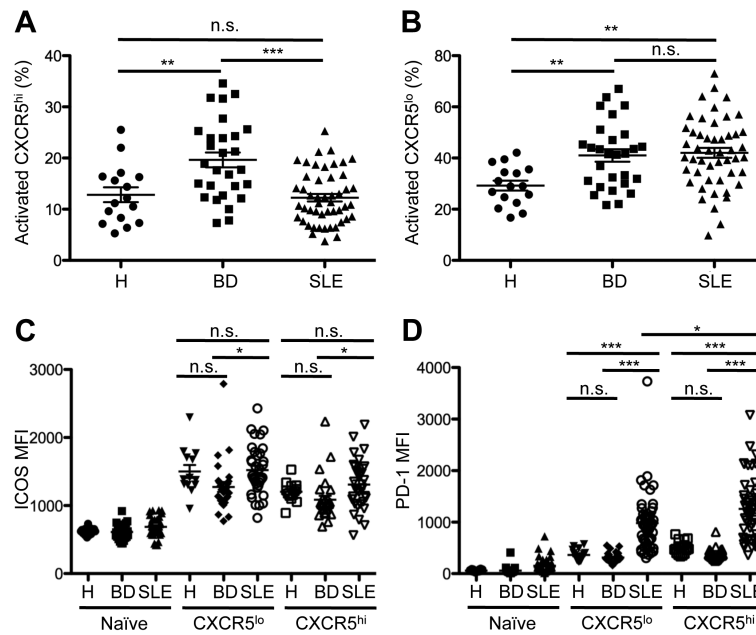


Figure 2.

Expression of the Tfh-cell surface markers CXCR5, PD-1, and ICOS on circulating CD4⁺ T lymphocytes. **A and B**, Percentage of activated CD45RA⁻CXCR5^{hi} (**A**) and CD45RA⁻CXCR5^{lo} (**B**) cells among the circulating CD4⁺TCRβ⁺ population in the blood of SLE patients ($n = 49$), in comparison to healthy controls (H; $n = 16$) and BD ($n = 28$) patients. **C and D**, MFI of ICOS (**C**) and PD-1 (**D**) expression on CXCR5^{hi} and CXCR5^{lo} CD4⁺ T cells from the blood of SLE patients compared to patients with BD and healthy controls. Data points represent individual subjects; horizontal lines represent the mean values with standard error of the mean (One-way ANOVA * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

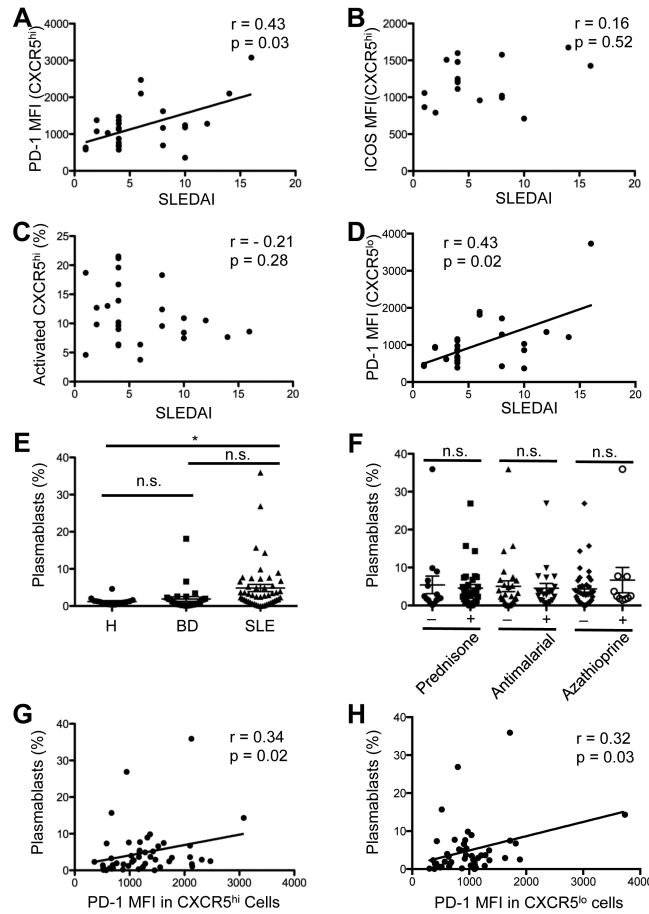


Figure 3. Association between Tfh-cell surface markers, SLEDAI, and circulating plasmablasts. **A and B**, Correlation between the MFI of PD-1 (**A**) and ICOS (**B**) on circulating CXCR5^{hi} cells and SLEDAI (**A**, Spearman $r = 0.43$, $p = 0.03$ and **B**, Spearman $r = 0.16$; $p = 0.52$). **C**, Association between percentage of CXCR5^{hi} cells among circulating CD4⁺TCRβ⁺ cells and SLEDAI (Spearman $r = -0.21$, $p = 0.28$). **D**, Correlation between MFI of PD-1 on the circulating CXCR5^{lo} subset and SLEDAI (Spearman $r = 0.43$, $p = 0.02$). **E**, Percentage of circulating CD19⁺IgD⁻CD38⁺ plasmablasts among total B cells in the blood of SLE patients ($n = 49$), healthy controls (H; $n = 16$), and BD ($n = 28$) patients (One-way ANOVA, $*p < 0.05$). **F**, Percentage of circulating plasmablasts in patients with SLE, arranged according to therapy (Student's t -test, n.s. = non significant). Horizontal lines represent the mean values with standard error of the mean. **G and H**, Correlation between percentage of circulating plasmablasts among circulating B cells in SLE and the PD-1 MFI on activated CXCR5^{hi} and CXCR5^{lo} cells, respectively (**G**, Spearman $r = 0.34$, $p = 0.02$; **H**, Spearman $r = 0.32$, $p = 0.03$). Data points represent individual subjects.

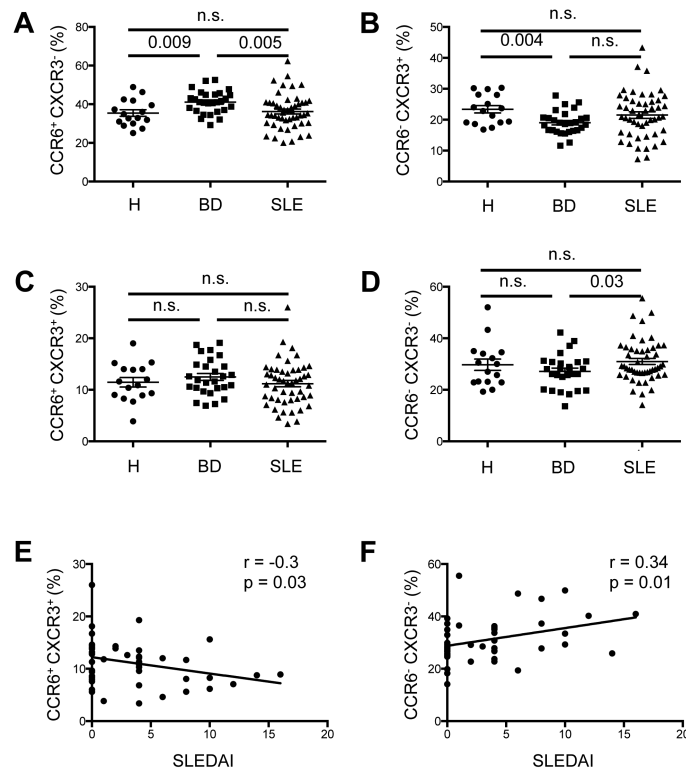


Figure 4. Analysis of CXCR5^{hi} Th-cell subsets and correlation with SLEDAI
A-D, Percentage of CCR6⁺CXCR3⁻ (**A**), CCR6⁻CXCR3⁺ (**B**), CCR6⁺CXCR3⁺ (**C**), and CCR6⁻CXCR3⁻ (**D**) cells among the circulating CD45RA⁻CXCR5^{hi} CD4⁺ population in SLE patients ($n = 49$), in comparison to healthy controls (H; $n = 16$) and BD ($n = 28$) patients. (Student's t -test, n.s. = non significant). Horizontal lines represent the mean values with standard error of the mean. **E and F**, Correlation between percentage of CCR6⁺CXCR3⁺ (**E**), and CCR6⁻CXCR3⁻ (**F**) cells among circulating, activated CD45RA⁻CXCR5^{hi} CD4⁺ cells and SLEDAI (**E**, Spearman $r = -0.3$, $p = 0.03$; **F**, Spearman $r = 0.34$, $p = 0.01$). Data points represent individual subjects.

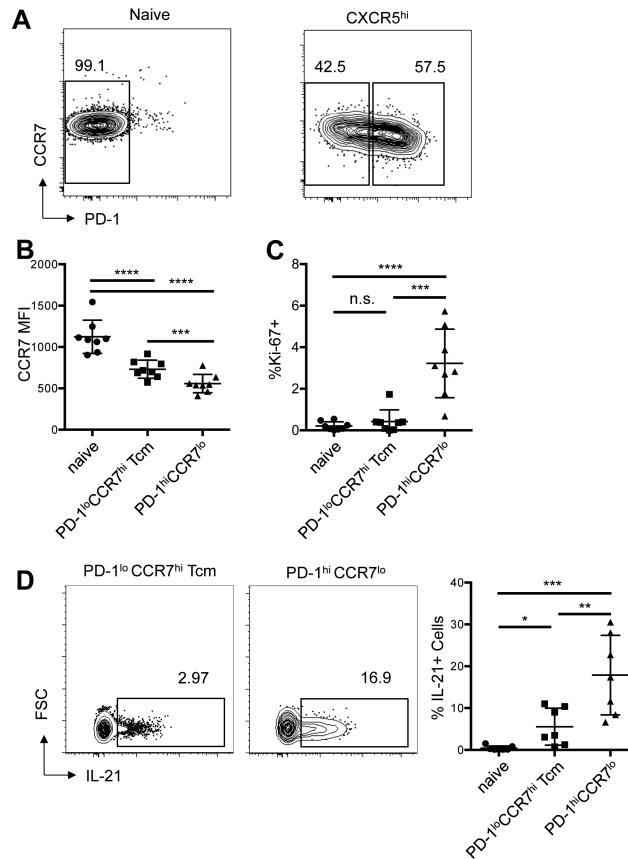


Figure 5.

Circulating CXCR5^{hi}ICOS^{hi}PD-1^{hi} cTfh-like cells in SLE are dividing and robustly produce IL-21, in comparison to CXCR5^{hi} central memory (Tcm) cells. **A**, Contour plots of CCR7 and PD-1 expression on naïve (left panel) and activated CD45RA⁻CXCR5^{hi} (right panel) cells in the blood of a representative patient with SLE, demonstrating the gating strategy to determine PD-1^{lo} Tcm and PD-1^{hi} cells. **B and C**, The MFI of CCR7 (**B**) and the percentage of Ki-67⁺ cells (**C**) in each of the indicated subsets (Student's *t*-test ****p* < 0.001, *****p* < 0.0001). **D**, Contour plots of intracellular IL-21 staining in circulating CXCR5^{hi}PD-1^{lo} Tcm populations and CXCR5^{hi}PD-1^{hi} cTfh-like populations from a representative patient with SLE (left and center panels) and the percentage of IL-21⁺ cells among seven SLE patients (right panel), compared to that among naïve CD45RA⁺ cells. Horizontal lines represent the mean values with standard deviation of the mean (right panel) (Student's *t*-test **p* < 0.05; ***p* < 0.01; ****p* < 0.001).

Table 1Mean fluorescence intensity (MFI) of PD-1 staining on CXCR5^{hi} cells and disease features

Current Disease	Absence of Disease Feature (MFI)	Presence of Disease Feature (MFI)	p-value
Proteinuria	1217	1380	0.4166
Hematuria	1211	1662	0.1086
Proteinuria + Hematuria	1199	1911	0.0199 *
Pyuria	1200	2590	0.0007 ***
Arthritis	1281	988.3	0.3501
Anti-dsDNA	1169	1891	0.0041 **
Low complement	1173	1862	0.0064 **
Past Disease			
Skin	1086	1286	0.4168
Joint	1523	1197	0.1387
Renal	1297	1222	0.6627
CNS	1295	1062	0.3150
Vasculitis	1226	1343	0.5498
Hematological	1246	1269	0.8909
Serositis	1087	1551	0.0070 **

Student's *t*-test* $p < 0.05$,** $p < 0.01$,*** $p < 0.001$