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Altered Circulating Follicular Helper T Cell Phenotype In Systemic Lupus Erythematosus

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ALTERED CIRCULATING FOLLICULAR HELPER T CELL PHENOTYPE IN SYSTEMIC LUPUS ERYTHEMATOSUS.

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Autoreactive B cells in Systemic Lupus Erythematosus (SLE) undergo autoantigen selection, suggesting a requirement for germinal center follicular helper T (Tfh) cells in their maturation. However, evidence for dysregulation of Tfh cells in SLE and their contribution to disease activity remains unclear. Recently, blood $CXCR5^{hi}$ CD4 T cells, a heterogeneous pool consisting of functionally distinct Th1-, Th2-, Th17-like subsets, have been proposed to be the circulating counterpart of Tfh cells. The purpose of this study was to examine the hypothesis that Tfh dysregulation, as reflected in altered phenotype of blood $CXCR5^{hi}$ population, was associated with SLE disease activity. Blood samples from 49 clinically well-characterized SLE patients, 28 Behçet's disease (BD) patients as autoimmune disease control and 16 healthy controls were included. Expression of key Tfh surface receptors (ICOS, inducible T-cell co-stimulator; PD-1, programmed cell death protein-1) as well as composition of T helper subtypes within the circulating $CXCR5^{hi}$ compartment were enumerated by flow-cytometry. The phenotype of blood $CXCR5^{hi}$ cells was correlated with clinical history and B cell phenotype. SLE patients had significant expansion of circulating Tfh-like CD4 T cells ($CXCR5^{hi}ICOS^{hi}PD\text{-}1^{hi}$) when compared to controls ($p < 0.001$). Interestingly, PD-1 mean florescence intensity (MFI) was markedly elevated in blood $CXCR5^{hi}$ subset of SLE patients when compared to controls ($p < 0.001$). PD-1 MFI in the $CXCR5^{hi}$ population correlated significantly with SLE disease activity index

(SLEDAI; Spearman r = 0.43, p = 0.03), blood plasmablast expansion (Spearman r = 0.34, p = 0.02), and high anti-dsDNA antibody titers (p = 0.004). Blood $CXCR5^{hi}PD\text{-}1^{hi}$ cells phenotypically resembled pre- or post-germinal center Tfh cells, with increased percentage of IL-21 producers (p = 0.02), lower CCR7 MFI (p = 0.03) when compared to $CXCR5^{hi}PD\text{-}1^{lo}$ subset, and low Bcl-6 expression. Compared to BD patients, SLE patients had an increase in the $CXCR5^{hi}$ Th2 subset ($p < 0.05$) and a decrease in the Th17 ($p < 0.001$) subsets. The expansion of the $CXCR5^{hi}$ Th2 subset was also positively associated with SLEDAI scores. Our results demonstrate that altered phenotype and subset composition of blood Tfh-like cells is correlated with disease activity in lupus patients, suggesting a potential role of GC Tfh dysregulation in the pathogenesis of human SLE.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease marked by immune complex-mediated tissue injury in multiple vital organs[1]. The clinical manifestations and the underlying immunoregulatory factors that contribute to disease expression are diverse[2]. Such heterogeneity poses a significant challenge for both clinical management and development of targeted therapeutics. Thus, there is a need to identify the common pathogenic cellular pathway that leads to lupus disease activity in an effort to define rational therapeutic targets.

The presence of autoantibodies is a common phenotype of SLE and their pathological role in lupus has been confirmed in animal models and clinical studies[3]. The collaboration between germinal center (GC) CD4 T cells and B cells, via processes such as high-affinity B cell selection and B cell memory induction, is central to the production of autoantibodie[4,5]. This is evidenced by the fact that pathogenic autoantibodies in SLE are high-affinity, somatically mutated, and Ig-switched antibodies that have undergone selection by T cells[6-8]. In addition, spontaneous formation of GCs has been documented in a mouse model of SLE[9]. Furthermore, intensive GC activity has been shown in patients with active lupus nephritis[10]. Taken together, these data point to a vital role of GC T cells in the pathogenesis of SLE.

Follicular helper T (Tfh) cells have been established as the specialized T cell subset responsible for the development of B cell response in the GC[11]. Tfh cells are positioned in the B cell follicles of secondary lymphoid organs and are identified by a combination of cellular markers, including CXCR5, the chemokine receptor that enables the migration of Tfh cells toward B cell follicles[12,13]; ICOS, a co-stimulatory

molecules important for the helper function of Tfh cells[14]; and PD1, a surface molecules classically known to provide inhibitory signals to the T cells[15], but which also regulate GC B cell survival[16]. Functionally, Tfh cells also secrete key cytokines that promote growth, differentiation, and antibody class-switching of B cells, such as IL-4 and IL-21[16-18]. Importantly, the expansion of Tfh cells is associated with the development of systemic autoimmunity in animal studies[19,20]. The expansion of Tfh cells has also been causally linked to the abundance of GCs, production of autoantibodies, and end-organ damage in a mouse model of SLE[21]. Thus, in animal models of lupus, Tfh cells are centrally involved in the cellular pathway that lead to clinical disease.

While the involvement of Tfh cells in animal models of SLE has been shown, evaluation of Tfh cells in human SLE has been limited by the inability to routinely study secondary lymphoid tissues in patients. Recently, however, studies have shown that circulating CXCR5^{hi} CD4 T cells are circulating counterparts of Tfh cells[22]. In ICOS- and CD40L-deficient patients, there are impaired GC formations and the absence of circulating CXCR5^{hi} subset[23]. Conversely, expansion of blood CXCR5^{hi} population has been noted post-immunization in humans[24]. In another recent study, CXCR5^{hi} subset is shown to efficiently induce naïve B cell differentiation and Ig type switch via secretion of key Tfh cytokines *in vitro*, demonstrating that they indeed share functional properties with Tfh cells[25]. Thus, blood CXCR5^{hi} CD4 T cells appears to be an attractive biomarker to study GC Tfh dysregulation in lupus.

Recently, the expansion of blood CXCR5^{hi}ICOS^{hi} or CXCR5^{hi}PD1^{hi} subset has been found in a subset of SLE patients with severe end-organ involvement[26]. However, the relationship between blood CXCR5^{hi} population and SLE disease activity has not been demonstrated. In addition, CXCR5^{hi} CD4 T cells appear to be heterogeneous and can be functionally grouped into Th1-, Th2-, and Th17-like cells[25]. Importantly, CXCR5^{hi} Th2 and Th17, but not Th1, subset have the capability to provide B cell help, and the skewed composition of CXCR5^{hi} CD4 T cell subsets may contribute to autoimmunity[25]. Whether the composition of blood CXCR5^{hi} cells in human SLE is altered remains unknown. In an effort to elucidate the involvement of Tfh cells in SLE disease activity, we examined the surface phenotype and subset heterogeneity of blood CXCR5^{hi} cells of SLE patients. We further correlated altered CXCR5^{hi} cell phenotype with clinical history, SLE disease activity, and pathological B cell activity. Lastly, we evaluated key Tfh cytokine secretion, gene expression and surface molecules of blood CXCR5^{hi} cells in order to establish the functional relationship between blood CXCR5^{hi} cells and GC Tfh cells.

THESIS HYPOTHESIS

We hypothesized that the phenotype and subset composition of circulating Tfh-like CXCR5^{hi} CD4 T cells are altered in human SLE, and that such alterations are associated with disease activity and pathological B cell activity, supporting a potential role of Tfh cells in the pathogenesis of SLE.

METHODS

Study populations. We analyzed blood samples from two adult cohorts. The first, from the Faculdade de Medicina, 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 Table 1. We also studied samples from 9 patients with SLE (8 female and 1 male; median age 40 years, range 26-63 years, 4 Caucasian, 5non-Caucasian) from Yale-New Haven Hospital (YNHH), with their data shown in Figure 4. SLE patients fulfilled American College of Rheumatology (ACR) criteria with disease activity assessed by the SLE Disease Activity Index, whereas BD patients met BD criteria set on International Study Group for Behcet's Disease. Informed consent was obtained from all subjects. This study was approved by the institutional review committees of the Faculdade de Medicina, 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 CD27, PE-conjugated PSGL1 and CD4, PE-Cy7-conjugated PD-1, CCR7, and IgD, Alexa Fluor 647-conjugated CXCR5 and IL-21, Fluorescein isothiocyanate (FITC)-conjugated CD62L, APC-Cy7-conjugated CD19, PE-Cy5-conjugated CD38, and streptavidin-conjugated APC-Cy7 (all from Becton Dickinson (BD) PharMingen except

FITC-conjugated ICOS and PE-CY5 conjugated TCR (eBioscience). Fluorescence-labeled cells were collected by multiparameter flow cytometry (LSRFortessa or LSR II; BD), with exclusion of doublets by forward and side scatter and analyzed with FlowJo software (Tree Star, Ashland, OR). $CD45RA^-$ activated CD4 cells divided to $CXCR5^{hi}$ and $CXCR5^{lo}$ cells (Figure 1A, left panel), and circulating Tfh-like cells were identified by high expressions of PD-1 and ICOS among $CXCR5^{hi}$ cells (Figure 1B, top right quadrants of left and middle panel); the expression of each molecule gated on the basal expression of PD-1 (< 0.5%) and ICOS (< 0.5%) on naïve CD45RA+ CD4+ T cells from healthy control, and subsequent double positive population of $ICOS^{hi}PD\text{-}1^{hi}$ (< 0.1%) (Figure 1A, right panel).

Intracellular IL-21 staining. PBMC stained with surface markers were stimulated with PMA (50 ng/ml) and ionomycin (1 g/ml) for first 2 hours then in the presence of golgi plug (BD) for last 2 hours. Cells were fixed with BD CytoFix/CytoPerm and permeabilized with BD PERM/ Wash solution, followed by staining with anti IL-21. Stained cells were collected by multiparameter flow cytometry (LSR II; BD), with exclusion of doublets by forward and side scatter and analyzed with FlowJo software (Tree Star, Ashland, OR).

Statistical analysis. All data are presented as the mean ± 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 circulating cells with Tfh surface phenotype in SLE patients. $CXCR5^{hi}$ Tfh cells in secondary lymphoid tissues express PD-1 and ICOS, surface molecules central to their B cell helper function in the B cell follicles[27]. By contrast, previously activated $CXCR5^{hi}$ CD4 T cells in the circulation of normal individuals are typically $PD\text{-}1^{lo}$ and $ICOS^{lo}$, suggesting that while these cells may be capable of entry in to B cell follicles via CXCR5, they may be at a resting state[28]. To determine whether blood $CXC5^{hi}$ cells carry key Tfh surface phenotype in lupus patients, we examined the expression of PD-1 and ICOS in blood samples of 49 SLE patients, 28 BD patients (autoimmune disease control), and 16 healthy controls. We analyzed single $CD4^{+}TCR\beta^{+}$ cells, dividing them into naïve ($CD45RA^{+}$), $CXCR5^{lo}$ ($CD45RA^{-}CXCR5^{lo}$), and $CXCR5^{hi}$ ($CD45RA^{-}CXCR5^{hi}$) subsets, with additional gating on PD-1 and ICOS according to their expression level on the naïve subset (Figure 1A, left panel). Naïve $CD45RA^{+}$ T cells were $CXCR5^{lo}$ and express basal level of ICOS and PD-1 (Figure 1A, right panel). Consistent with previous reports, majority of blood $CXCR5^{hi}$ cells are $PD\text{-}1^{lo}ICOS^{lo}$ in healthy donors (Figure 1 B, right panel, representative example; Figure 1C)[28]. Compared with healthy control, there was a significantly higher percentage of blood $CXCR5^{hi}$ cells in lupus patients carrying GC Tfh surface phenotype, eg. $PD\text{-}1^{hi}ICOS^{hi}$ ($p < 0.001$; Figure 1B, middle panel, blood $CXCR5^{hi}$ cells, representative example; Figure 1B, right panel, Tonsil GC Tfh cells; Figure 1C). In BD patients, there was no increased percentage of blood $CXCR5^{hi}$ cells expressing high levels of PD-1 and ICOS, suggesting that the presence of circulating Tfh-like $CXCR5^{hi}PD\text{-}1^{hiI}COS^{hi}$ cells in lupus patients is not merely a function of systemic

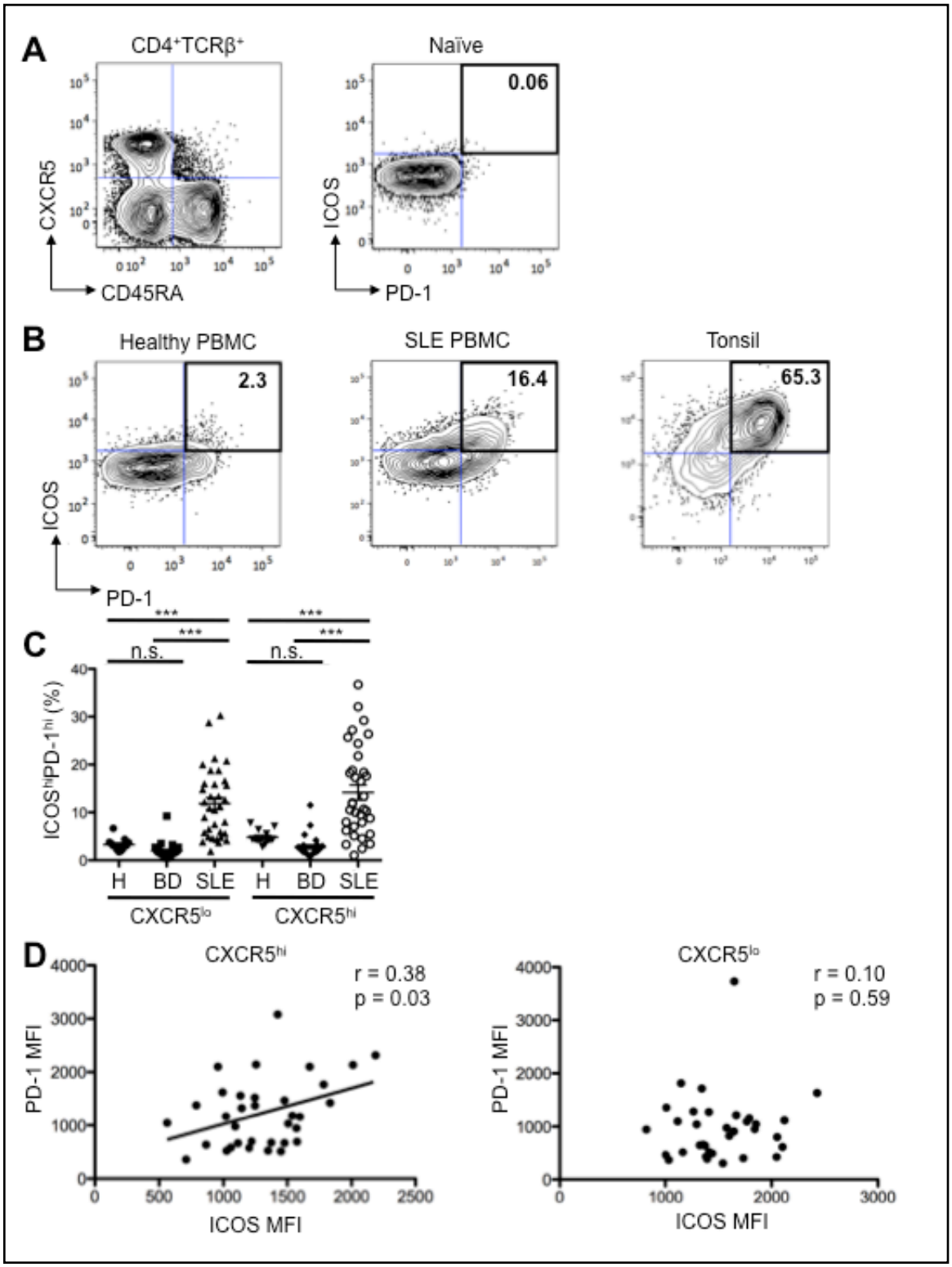

Figure 1. Prevalence of circulating cells with Tfh phenotype in SLE, BD, and healthy controls.

autoimmunity (Figure 1C). We also noted a significant increase in the percentage of ICOS^{hi}PD-1^{hi} cells within the CXCR5^{lo} subset in lupus patients ($p < 0.001$; Figure 1C). However, there was a disassociation of PD-1 and ICOS MFI in the CXCR5^{lo} subset (Pearson $r = 0.10$, $p = 0.59$; Figure 1D left panel), whereas there was a significant correlation of their expression in the CXCR5^{hi} subset (Pearson $r = 0.38$, $p = 0.03$; Figure 1D right panel). Thus, CXCR5^{hi} subset, but not CXCR5^{lo} subset, resembles GC Tfh cells per analysis of surface receptor phenotype.

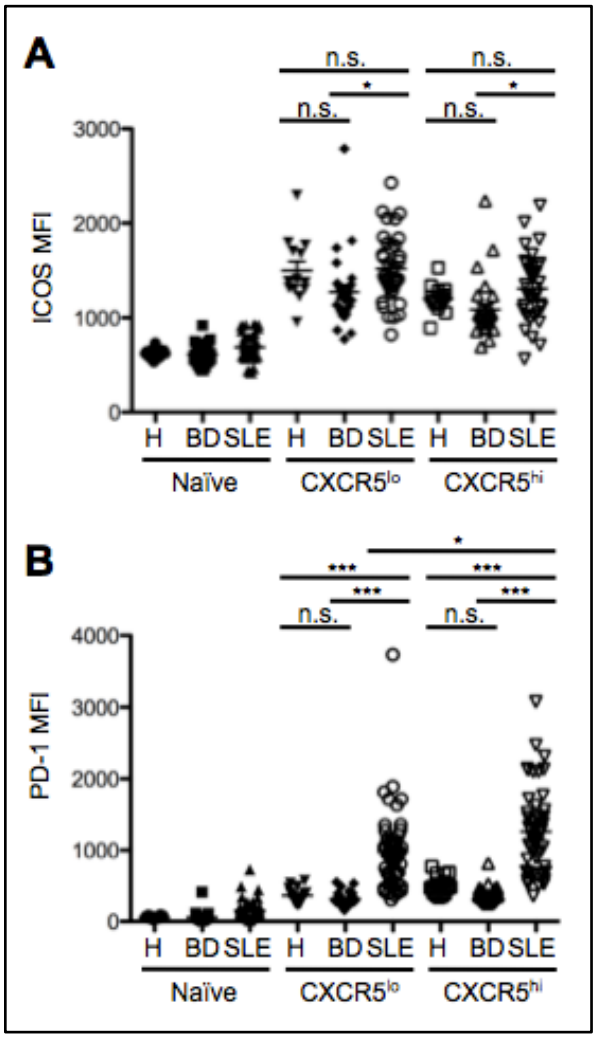

Figure 2. Expression of Tfh surface receptors in differential subsets of circulating CD4 T cells in SLE, BD, and healthy controls.

We next asked if a single Tfh-related surface receptor was selectively upregulated in lupus patients. ICOS is a co-stimulatory molecule important for the helper function of Tfh cells[14]. Previous studies have found an increase in circulating $CD4^{+}ICOS^{hi}$ lymphocytes, although their number varied significantly depending on how the fraction was defined and the SLE cohort studied[29-31]. In our SLE cohort, we did not find a significant increase in ICOS MFI in blood $CXCR5^{hi}$ cells ($p > 0.05$; figure 2A).

PD-1 is another key Tfh surface receptor, and it plays an integral role in the formation and affinity of plasma cells in the context of GC processes[16]. Interestingly, we found a drastic increase in PD-1 MFI in the $CXCR5^{hi}$ population as compared to both healthy control and BD patients ($p < 0.001$; Figure 2B). PD-1 expression was also increased in the $CXCR5^{lo}$ subset, but its PD-1 MFI was significantly lower than those of $CXCR5^{hi}$ subset (Figure 2B).

Taken together, we have found a significant expansion of blood $CXCR5^{hi}$ cells that are $PD\text{-}1^{hi}ICOS^{hi}$, akin to GC Tfh cells, in lupus patients. PD-1, a molecule known for its role in GC B cell survival, but not ICOS, was selectively upregulated in the $CXCR5^{hi}$ subset in lupus compared to healthy controls.

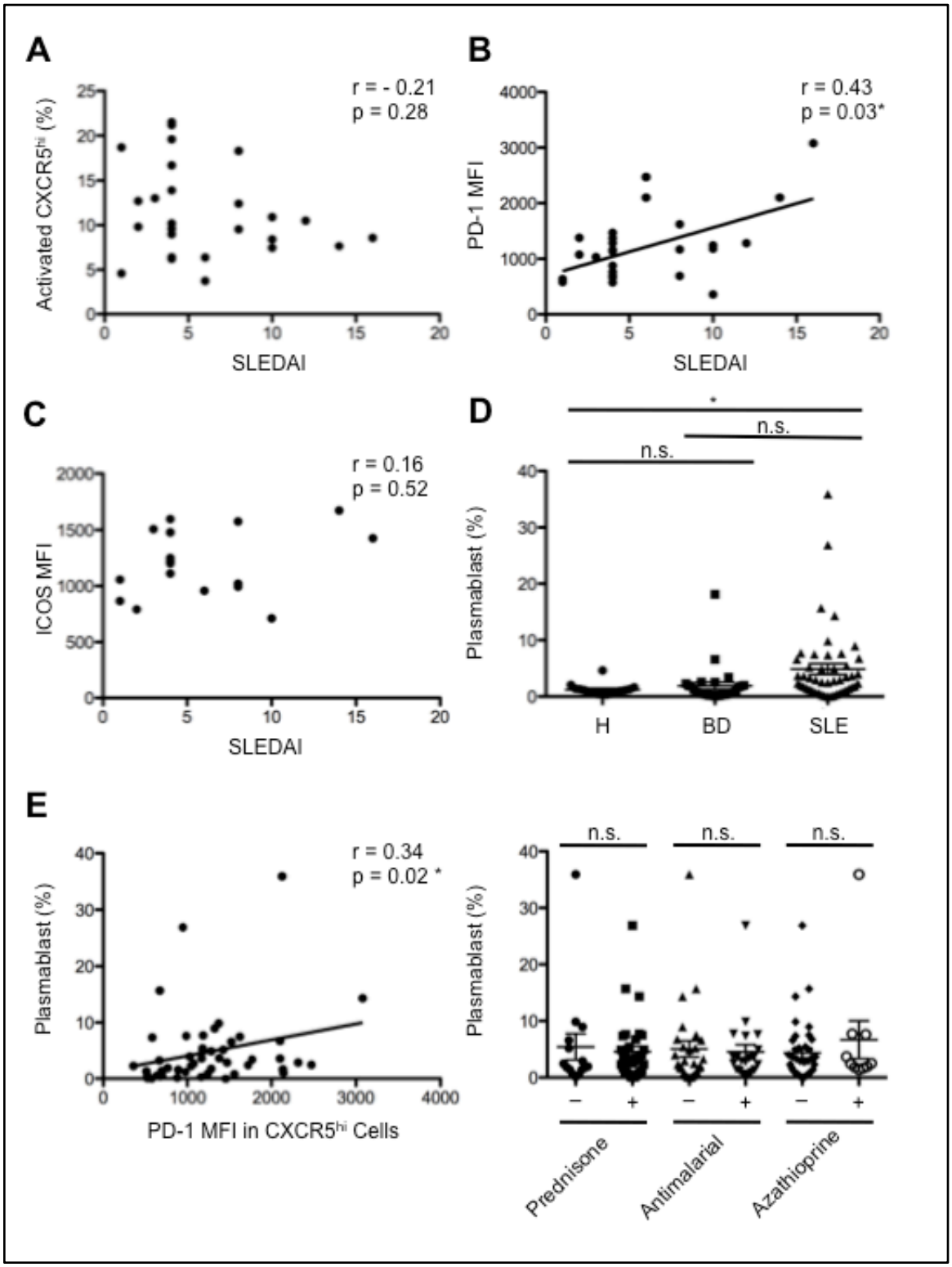

Figure 3. PD-1 expression on circulating CXCR5^{hi} subset correlates with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and expansion of circulating plasmablasts.

Correlation between PD-1 expression on blood CXCR5^{hi} cells and disease activity in SLE. We next asked if altered phenotype of blood CXCR5^{hi} cells, the circulating counterpart of Tfh cells, correlated with SLE disease activity. We first examined the relationship between the expansion of CXCR5^{hi}PD-1^{hi}ICOS^{hi} cells and SLE disease activity index (SLEDAI); however, we did not find a significant correlation between the two variables (Spearman's r = 0.26, p = 0.19; data not shown). Similarly, we did not find significant correlations between the percentages of CXCR5^{hi}PD-1^{hi}ICOS^{hi} cells and organ involvement, as well as aberrant B cells activation. In addition, we did not find a correlation between the percentage of CXCR5^{hi} cells in CD4 T cells and SLEDAI (Spearman's r = -0.21, p = 0.28; Figure 3A).

We then asked whether differential expression of key Tfh surface receptors on blood CXCR5^{hi} cells is correlated with disease activity in human lupus. In the cohort of SLE patients with active disease, we found a significant correlation between PD-1 MFI in blood CXCR5^{hi} subset and SLEDAI scores (Spearman r = 0.43, p = 0.03; Figure 3B). Elevated PD-1 may merely reflect T cells activation[32] that is common in active SLE; however, this did not appear to be the case, as ICOS, a marker of activated T cells, and the patients' SLEDAI was not correlated (Spearman r = 0.16, p = 0.52; Figure 3C). Moreover, there was no relationship between the percentage of activated CD45RA^{-} CD4 T cells and SLEDAI (data not shown).

Given the association between PD-1 MFI on CXCR5^{hi} cells and disease activity, we also asked if the former was linked to organ involvement. In patients with concurrent proteinuria and hematuria, compared 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 serositits (1551 vs. 1087, p = 0.007), PD-1 did not correlate with past organ injury, including skin, joint, kidney, CNS, and vasculature. These results in aggregate indicated that the PD-1 MFI on $CXCR5^{hi}$ cells correlated with disease activity, including renal inflammation. Consistent with this theme, we found that PD-1 MFI on the $CXCR5^{hi}$ CD4 T cell subset was increased in patients with elevated anti-dsDNA antibody titers, when compared to patients without such autoantibodies (1891 vs. 1169, respectively; p = 0.004), and elevated hypocomplementemic patients (1862 vs. 1173, p = 0.006), with the latter presumably reflecting consumption upon engagement by autoantibody-autoantigen immune complexes as a feature of active SLE[3].

Association between PD-1 MFI and expansion of circulating plasmablasts. The correlation between PD-1 on circulating $CD4^{+}CXCR5^{hi}$ T cells and anti-dsDNA antibody titers and presumed complement consumption suggested that elevated expression of this protein might also be correlated with aberrant B cell activation. PD-1 is also critical, among other proteins, for Tfh-directed GC maturation and plasma cell genesis[16]. We therefore purified lymphocytes from patients and controls, staining them with CD19, IgD, and CD38 to assess the pool of the $IgD^{-}CD38^{hi}$ plasmablasts[33]. Consistent with previous reports[34], we found an increase in the percentage of circulating plasmablasts in SLE patients compared to healthy controls (Figure 3D). We also found that the PD-1 MFI among $CXCR5^{hi}$ cells positively correlated with the expansion of plasmablast in lupus patients (Figure 3E, left panel). Of note, the percentage of plasmablasts was similar between SLE patients regardless of treatment (Figure 3E, right panel).

Analysis of circulating CXCR5^{hi}PD-1^{hi} cells in human SLE patients. Since the presence circulating CXCR5^{hi} cells carrying high levels of PD-1 strongly correlated with disease activity and pathological B cell activity in lupus, we next asked how the CXCR5^{hi}PD-1^{hi} cells are differ from the CXCR5^{hi}PD-1^{lo} cells, and whether they exhibit key characteristics of GC Tfh cells. IL-21 is a key cytokine secreted by Tfh cells, leading to differentiation, proliferation, isotype switching of B cells[17,18]. We therefore examined the presence of IL21-producing cells in circulating CXCR5^{hi}PD-1^{hi} CD4 T cells of lupus patients. We sought CD4+CD45RA-CXCR5^{hi} cells and divided them into PD-1^{lo} and PD-1^{hi} subset to measure the percentage of IL-21 producing cells in each population (Figure 4A, left panel; representative example). In lupus patients, we found a significant higher proportion of IL21+ cells in the CXCR5^{hi}PD-1^{hi} subset compared to CXCR5^{hi}PD-1^{lo} subset ($p = 0.02$; Figure 4A, right panel). The secretion of IL21 suggests that blood CXCR5^{hi}PD-1^{hi} cells in lupus patients are functionally related to bona fide Tfh cells in their capability to provide B cell-help signals.

Previously, circulating CXCR5^{hi} CD4 T cells in healthy donors were found to have a central memory phenotype, with high expression level of CCR7, which is required for cell entry into secondary lymphoid organs[28]. In contrast, GC Tfh cells down-regulate CCR7 in order to achieve proper follicular positioning. We therefore asked whether blood CXCR5^{hi}PD-1^{hi} cells in lupus carried a central memory phenotype[28] or a GC Tfh-like phenotype by analyzing their level of CCR7 expression. We compared CCR7 MFI of CXCR5^{hi}PD-1^{hi} cells and CXCR5^{hi}PD-1^{lo} cells in the form of its relative expression to CCR7 MFI on naïve CD45RA^{+}CD4 T cells

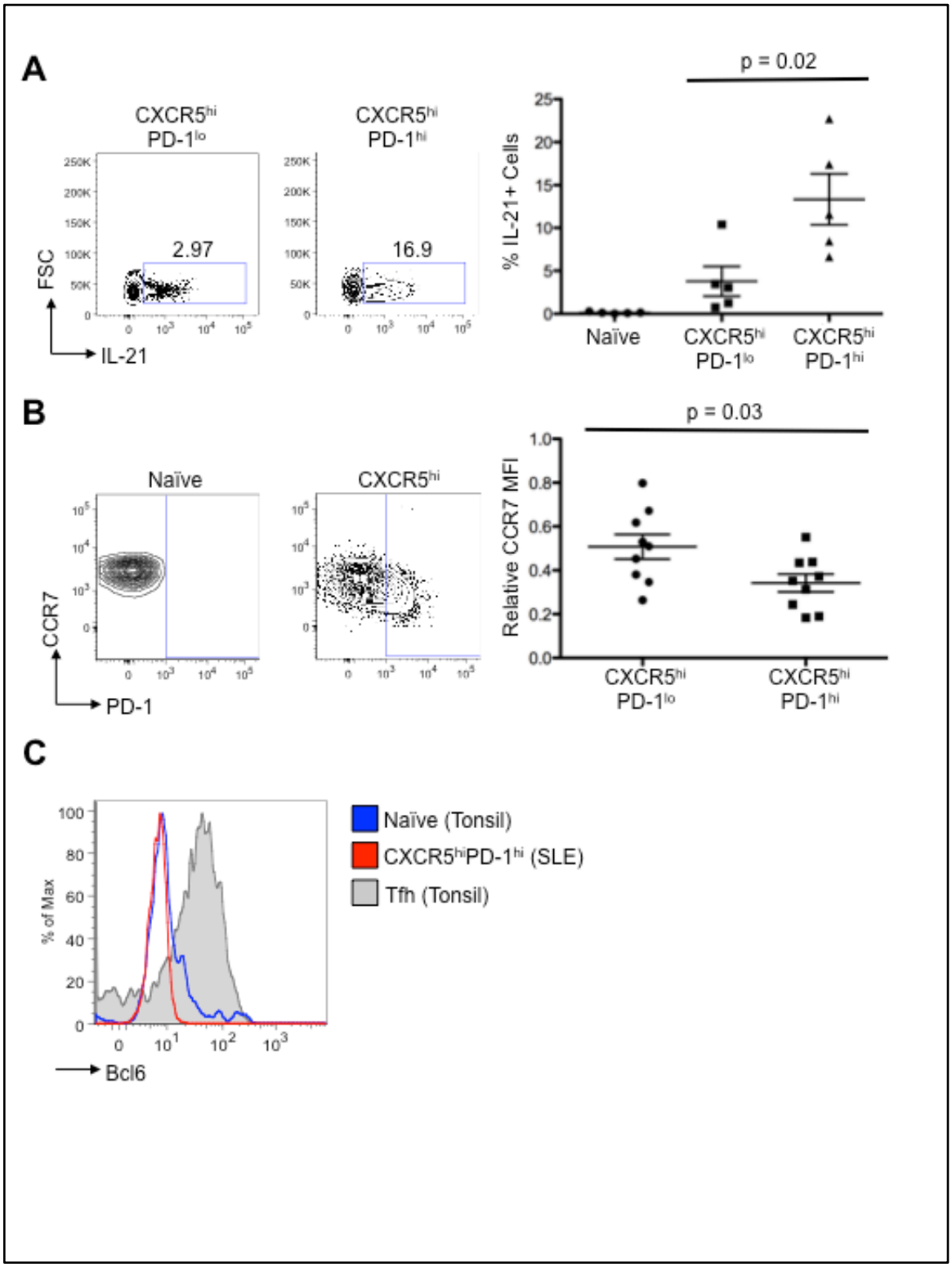

Figure 4. Functional and phenotypic analysis of circulating $CXCR5^{hi}PD\text{-}1^{hi}$ cells.

(representative sample, figure 4B, left panel). Interestingly, we found a significant lower CCR7 expression in $CXCR5^{hi}PD\text{-}1^{hi}$ cells when compared to $CXCR5^{hi}PD\text{-}1^{lo}$ cells. Thus, blood $CXCR5^{hi}PD\text{-}1^{hi}$ cells do not resemble the previously described circulating $CXCR5^{hi}$ central memory population. Instead, it more resembles active Tfh cells in secondary lymphoid organ based on memory marker analysis.

Bcl-6 is the key transcriptional factor that drives Tfh formation[35-37]. It is highly expressed in GC Tfh cells[38,39]. In addition, its overexpression is sufficient to upregulate CXCR5, PD-1, ICOS, and downregulate CCR7 *in vitro* and *in vivo* [35,36]. Therefore, we assessed Bcl-6 expression in blood $CXCR5^{hi}PD\text{-}1^{hi}$ cells of lupus sample as compared to naïve $CD45RA^{+}$ CD4 T cells and $CXCR^{hi}PD\text{-}1^{hi}ICOS^{hi}$ Tfh cells from Tonsils of a non-lupus patient. We found that blood $CXCR5^{hi}PD\text{-}1^{hi}$ cells in lupus express the basal level of bcl-6 at a level similar to that of naïve CD4 T cells (representative sample, Figure 4C).

Taken together, our data supports a model in which follicular helper T cell phenotype is altered in human systemic lupus erythematosus (Figure 5). In such model, presentation of autoantigen by dendritic cells (DC) to T cells leads to the differentiation of T helper (Th) cells and follicular helper T precursors (pre-Tfh) (1, Figure 5). Tfh-destined cells migrate to border of T-cell zones and B-cell follicles, where they contact autoantigen-activated B cells (2, Figure 5). Subsequent migration of both subsets deeper into the follicle leads to germinal center (GC) formation (3, Figure 5). GC Tfh cells are notable for high CXCR5, PD-1, and Bcl6 expression, as well as production of IL-21. In

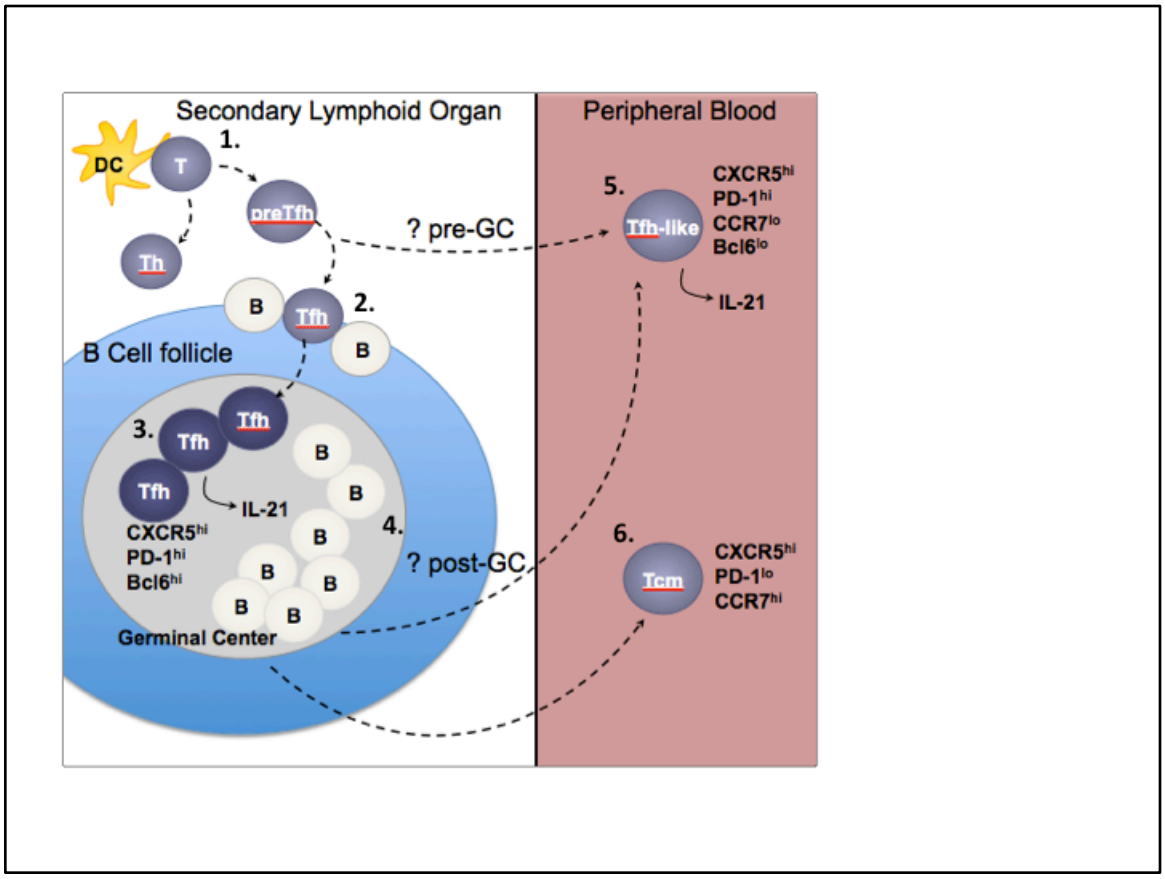

Figure 5. Model of altered follicular helper T cell phenotype in systemic lupus erythematosus.

the GC, Tfh cells promote class switching and affinity selection of B cells via secretion of cytokine (eg. IL-21) and expression of cell bound molecules (eg. PD-1). The result is the production of high-affinity autoantibody-secreting plasma cells, leading to pathology and disease activity (4, Figure 5). In parallel, there is an expansion of Tfh-like cells in the peripheral blood (5, Figure 5). Akin to Tfh cells, these Tfh-like cells also express high levels of CXCR5, PD-1, and are enriched with IL-21 producers. The presence of $CXCR5^{hi}PD\text{-}1^{hi}$ Tfh-like cells in blood is associated with disease activity, plasmablast expansion, and autoantibody production. Unlike GC Tfh cells, circulating Tfh-like cells have low levels of Bcl6, indicating that they may have relocated to the periphery pre- or post-germinal center reaction. Importantly, these circulating Tfh-like cells express low

level of CCR7, which differentiate them from previously described circulating central memory T cells with high level of $CXCR5^{28}$ (6, figure 5).

Differential composition of Tfh subsets in human SLE. Recently, human blood $CXCR5^{hi}$ CD4 T cells was found to consist of three subsets (Th1, Th2, and Th17), based on their cytokine production and chemokine receptor profile[25]. Notably, Th2 and Th17, but not Th1, $CXCR5^{hi}$ CD4 T cells secrete IL-21 and promote differential antibody secretion by naïve B cells *in vitro*[25]. Consistent with previous findings, we found that a portion, but not all, of $CXCR5^{hi}$ CD4 T cells were IL-21 producers (Figure 4A). We therefore asked if there is another dimension of Tfh dysregulation in human SLE, namely, if the blood $CXCR5^{hi}$ subset composition was different among lupus patients and our two control groups. We gated on circulating $CD4^{+}TCR\beta^{+}CD45RA^{-}CXCR5^{hi}$ lymphocytes, using chemokine receptors (CXCR3, CCR4, and CCR6) to identify subsets, with Th1 cells defined as $CXCR3^{hi}CCR4^{-}CCR6^{lo}$, Th2 as $CXCR3^{lo}CCR4^{hi}CCR6l^{o}$, and the Th17 subset as $CXCR3^{lo}CCR4^{lo}CCR6^{hi}$ (Figure 6A)[25,40]. Where as lupus patients had a reduction of Th1 $CXCR5^{hi}$ cells compared to healthy donors, and a decrease in the Th17 subset compared to BD patients (Figure 6B, left and right panel, respectively), we found expansion of the $CXCR5^{hi}$ Th2 subset compared to BD patients, with a trend toward an increase compared to healthy controls (Figure 6B, middle panel). Consistent with the latter finding was the positive association between the frequency of $CXCR5^{hi}$ Th2 cells and patients' SLEDAI, an association that is not clearly observed with the Th1 and Th17 populations (Figure 6C).

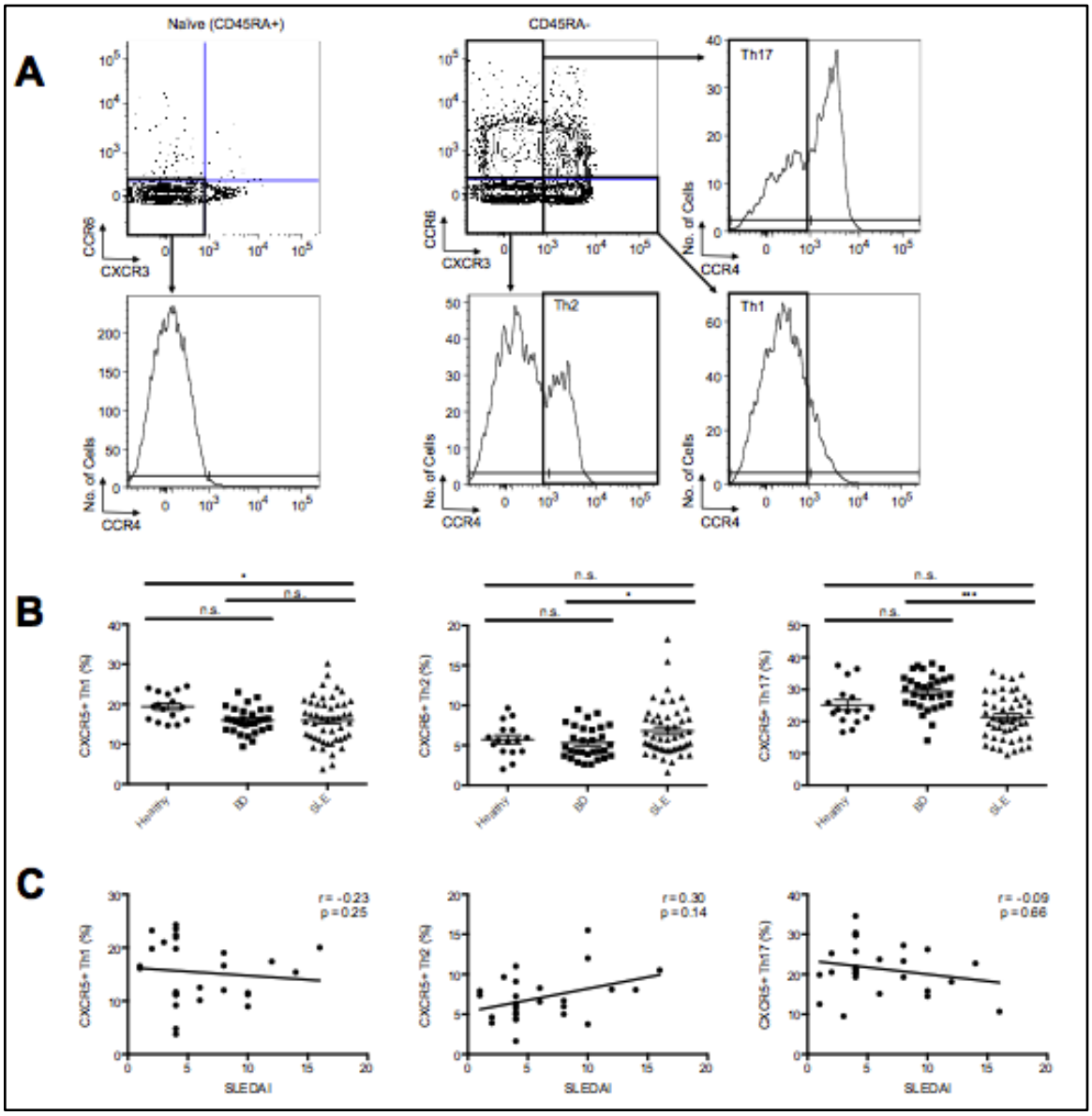

Figure 6. Composition of circulating CXCR5^{hi} subsets and its relationship with SLE disease activity.

DISCUSSION

SLE is an autoimmune disease characterized by the production of autoantibodies. The pathogenic antibodies in SLE are isotype-switched, suggesting the involvement Tfh cells in the GC response[8]. However, the involvement of Tfh cells in disease activity of human SLE remains unclear. Blood $CXCR5^{hi}$ cells are attractive biomarkers to study human Tfh dysregulation because they exhibit key functional and physical characteristics of Tfh cells and have been proposed to be their circulating counterparts[25]. In addition, expansion of blood $CXCR5^{hi}PD\text{-}1^{hi}$ and $CXCR5^{hi}ICOS^{hi}$ cells have been found in a subset of SLE patients with severe end-organ involvement[26].

$CXCR5^{hi}$ Tfh cells in secondary lymphoid tissues express high levels of PD-1 and ICOS, surface molecules central to their B cell helper function[27]. In contrast, blood $CXCR5^{hi}$ CD4 T cells in healthy individuals are $PD\text{-}1^{lo}ICOS^{lo}$, suggesting that while they are capable of entry into B cell follicles via CXCR5, they may not be actively carrying out Tfh functions[28]. Here, we report a significant increase of $CXCR5^{hi}PD\text{-}1^{hi}ICOS^{hi}$ cells in SLE patients as compared to healthy controls. Such expansion was not seen in Behçet's disease, demonstrating that it was not merely a function of systemic inflammatory disorder, but a distinctive phenotype in human SLE. The presence of such circulating Tfh-like cells likely reflect germinal center dysfunction, a hallmark of murine lupus[19-21], and one likely present in human SLE, as evidenced by studies demonstrating abnormal GC B cell selection[41] and circulating GC B cells in patients with active disease[42-44].

We have found that the combination of high CXCR5 and high PD-1 expression effectively identifies circulating cells resembling pre- or post-GC Tfh cells in SLE

patients. High expression of CXCR5 implies that these cells are capable of being retained near B cells follicles in response to CXCL13 much like Tfh cells[13,27]. High expression of PD-1 suggests that these cells have the capability to regulate GC B cell survival[16]. These cells also express relatively higher amounts of ICOS as we found a significant positive correlation between ICOS and PD-1 expression in $CXCR5^{hi}$ CD4 T cells. The ICOS-ICOSL interaction between T cells and B cells has been shown to be central in T-dependent antibody response, Ig switching, and GC formation[45-47]. Importantly, blood $CXCR5^{hi}PD\text{-}1^{hi}$ cells are enriched with IL-21 producers when compared to $CXCR5^{hi}PD\text{-}1^{lo}$ cells. IL-21 is a hallmark cytokine produced by Tfh cells, and is important for the differentiation, proliferation, isotype-switching of B cells[17,18,48]. Thus, blood $CXCR5^{hi}PD\text{-}1^{hi}$ cells strongly resemble Tfh cells based on phenotypic and functional analysis. We also found that circulating $CXCR5^{hi}PD\text{-}1^{hi}$ cells expressed lower level of Bcl-6 when compared to GC Tfh cells[39,49]. This result is consistent with previous findings in blood $CXCR5^{hi}ICOS^{hi}$ Tfh-like cells of lupus patients[26]. Our result implies that blood $CXCR5^{hi}PD\text{-}1^{hi}$ cells may be pre-GC Tfh cells. Alternatively, Bcl-6 may have been down-regulated post-GC reaction in a manner analogous to decreased Bcl-6 expression level in post-GC B cells[50], suggesting that these cells are post-GC Tfh cells that have been dislocated into the periphery. The presence of these pre- or post-GC Tfh-like cells in the periphery strongly suggest that there is GC Tfh expansion or dysfunction in human SLE – a consistent finding in multiple animal models of SLE[19-21]. In the future, the relationship between blood $CXCR5^{hi}PD\text{-}1^{hi}$ cells and GC Tfh cells may be further clarified using, for instance, an IL-21 reporter mice system[51].

Importantly, the circulating CXCR5^{hi}PD-1^{hi} population in human SLE is distinct from the previously described blood CXCR5^{hi} central memory T (Tcm) cells[28]. We found that blood CXCR5^{hi}PD-1^{hi} cells expressed low levels of CCR7, a key chemokine receptor of memory T cells that control migration to secondary lymphoid organs[52]. Thus, they did not resemble CXCR5^{hi} Tcm cells phenotypically. The combination of high CXCR5 and low CCR7 also implies that these cells are more similar to Tfh cells, rather then Tcm cells, in their localization capability. This is because while CXCR5 expression is sufficient for localization to the T-B border, CXCR5 expression is not sufficient for Tfh cells to localize inside follicles[53]. In fact, high levels of CCR7 expression can block T cell migration to the B cell zone[54]. Thus, Tfh cells needs to downregulate CCR7 in order to migrate to T-B boundary region[55], a phenotype consistent with the circulating CXCR5^{hi}PD-1^{hi} cells in human SLE.

We have found that PD-1 MFI in blood CXCR5^{hi} cells correlated significant with disease activity in SLE patients with active disease. PD-1 MFI in CXCR5^{hi} cells is a composite value reflecting the shift from CXCR5^{hi}PD-1^{lo} Tcm cells to CXCR5^{hi}PD-1^{hi} pre- or post-GC Tfh cells. It also reflects PD-1 expression level in CXCR5^{hi}PD-1^{hi} cells. This raises the possibility that both the expansion of Tfh compartment and the degree of PD-1 expression on Tfh cells may contribute to SLE disease activity and thus play a role in lupus pathology.

With regards to the expansion of Tfh cells, such phenotype has already been causally linked to the abundance of GCs, production of autoantibodies, and end-organ damage in a mouse model of SLE[21]. The expansion of circulating Tfh-like cells has also been associated with severity of end organ damage in human SLE[26]. Our

results show for the first time that such phenotype is also associated with disease activity. A previous report has found no relationship between SLE disease activity and the expansion of Tfh-like $CXCR5^{hi}ICOS^{hi}$ cells[26]. The two studies differ in the use of PD-1 vs. ICOS to define blood Tfh-like cells. Here, we found that PD-1, but not ICOS, was significantly upregulated in $CXCR5^{hi}$ cells of SLE patients when compared to healthy controls and thus served as a better marker to differentiate Tfh-like cells from $CXC5^{hi}$ Tcm cells. In concordance, previous studies have found that the number of $CD4^{+}ICOS^{hi}$ cells varied significantly depending on how the fraction was defined and the SLE cohort studied[29-31]. Furthermore, consistent with the correlation between Tfh dysregualtion and SLE disease activity, we have found strong association between Tfh dysregulation, expansion of circulating plasmablast, production of autoantibodies, and ongoing kidney injury. Together, these findings suggest that expansion of Tfh cells are intimately involved in SLE disease flares, making them possible targets for monitoring or treating an increase in disease activity. Further validation with longitudinal studies will clarify their potential usefulness as biomarkers of disease activity and as a therapeutic target.

Our findings suggest that the degree of PD-1 expression on Tfh cells may also contribute to GC dysfunction and SLE disease activity. Indeed, PD-1 expression on Tfh cells appears to play an important role in regulating GC cell survival[16]. Classically, PD-1 is known as an inhibitory receptor associated with CD8 T cell exhaustion during chronic viral infection[15,56,57]. However, PD-1 is highly expressed on GC Tfh cells[54,58,59], and GC B cells express PD-L1 and elevated PD-L2[16]. Interestingly, mice deficient in PD-1 or PD-1 ligands had worsened B cell response, with increased GC cell death and

decreased Tfh cell cytokine production, such as IL-4 and IL21[16]. It is important to note that PD-1 can provide bidirectional signaling through PD1-PDL1 or PDL2 complexes[15], and PD-1 may provide direct inductive signals from GC Tfh to GC B cells or affect duration of T-B conjugates[55,60]. Though the loss of function of PD-1 has been studied in the context of GC reaction[16], conducting gain of function study in the future may help dissecting the mechanism between elevated PD-1 expression on Tfh-like cells and SLE disease activity. Of note, the upregulation of PD-1 on circulating CD4 T cells in SLE patients did not appear to be a reflection of generalized T cell activation as we did not find a correlation between the percentage of activated ($CD45RA^-$) CD4 T cells and PD-1 expression (data not shown). Elevated PD-1 also did not appear to be merely a function of systemic autoimmunity as there was no alteration of PD-1 expression in BD patients. Multiple factors may have contributed to elevated PD-1 expression in Tfh-like cells. First, there is increased IL-21 secretion in human SLE[61], which is able to induce PD-1 expression[62]. Second, there is increased type 1 interferon signal in SLE[63], which can enhance humoral immunity and promote isotype switching *in vivo*, suggesting the involvement of Tfh cells. IFN regulated-element has also been found upstream of PD-1 gene in mice and enhances PD-1 expression in CD4 T cells[64]. Lastly, elevated Tfh PD-1 may also be a reflection of repeated TCR stimulation via increased T-B contact in SLE[15].

We showed that there is an altered composition of circulating Tfh subsets in SLE. In both SLE and BD patients, there was an underrepresentation of $CXCR5^{hi}$ Th1 cells compared to healthy controls. A similar observation has been reported in patients with juvenile dermatomyositis[25]. As previously demonstrated, $CXCR5^{hi}$ Th1 cells lacked B helper activity[25], and therefore the expansion of non-Th1 Tfh cells may be a significant

contributing factor to pathogenic autoantibody production in SLE. Interestingly, there was differential expansion of $CXCR5^{hi}$ subsets in SLE vs. BD patients. SLE patients had significantly higher $CXCR5^{hi}$ Th2 cells compared to BD, while BD patients had significantly higher $CXCR5^{hi}$ Th17 cells compared to SLE. While both Th2 and Th17 Tfh subtypes help B cells and induce Ig switch, Th2 subset is better at inducing IgE production while Th17 subset is better at inducing IgA production[25]. Thus, the differential expansion of functional Tfh subset may shape the characteristics of antibody response in a disease specific manner.

In conclusion, our data show an expansion of circulating pre- or post-GC Tfh cells in human SLE. The expansion of Tfh compartment, along with elevated expression of PD-1, a key regulator of GC cell survival, is strongly associated with SLE disease activity. Thus, Tfh population may be an attractive candidate for the monitoring and treatment of SLE disease flares. We have also described, for the first time, the altered composition of Tfh subsets in SLE and revealed another dimension of Tfh dysregulation previously not appreciated. These data provide a basis to better elucidate the dynamics of pathological Tfh activity in human SLE.

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FIGURE LEGENDS

Figure 1. Prevalence of circulating cells with Tfh phenotype ($CXCR5^{hi}ICOS^{hi}PD\text{-}1^{hi}$) in blood samples of healthy controls (H; n = 16), patients with Behçet's disease (BD; n = 28), and patients with systemic lupus erythematosus (SLE; n = 49). **A,** Representative fluorescence-activated cell sorter (FACS) analysis of circulating lymphocytes, gated on viable $CD4^{+}TCR\beta^{+}$ cells (left panel), plus $CD45RA^{+}$ cells (naïve subset), $CD45RA^{-}CXCR5^{lo}$ cells ($CXCR5^{lo}$ subset), and $CD45RA^{-}CXCR5^{hi}$ cells ($CXCR5^{hi}$ subset). Representative expression of ICOS and PD-1 in naïve subset; value in naïve subset gate ($ICOS^{hi}PD\text{-}1^{hi}$ [black box]) is the percentage of viable lymphocytes (right panel). **B,** Representative FACS analysis of ICOS and PD-1 expression in the $CXCR5^{hi}$ subset from healthy peripheral blood mononuclear cells (PBMC; left panel), SLE PBMC (middle panel), and Tonsil (right panel). Values in the compartments (black box) are the percentages of $ICOS^{hi}PD\text{-}1^{hi}$ cells. **C,** Expansion of $ICOS^{hi}PD\text{-}1^{hi}$ cells in $CXCR5^{hi}$ and $CXCR5^{lo}$ subset in SLE patients, but not in BD patients or healthy controls. Statistical analysis was performed using One-way ANOVA (*** $p < 0.001$). **D,** Correlation between PD-1 and ICOS expression (mean fluorescence intensity; MFI) in $CXCR5^{hi}$ (Pearson $r = 0.38$, $p = 0.03$), but not $CXCR5^{lo}$ (Pearson $r = 0.10$, $p = 0.59$) subset. Data points represent individual subjects; horizontal lines represent the mean value with standard error of the mean.

Figure 2. Expression of follicular helper T cell (Tfh) surface receptors in circulating CD4+ lymphocytes of healthy controls (H), patients with Behçet's disease (BD), and patients with systemic lupus erythematosus (SLE). **A,** similar ICOS expression (MFI) in $CXCR5^{hi}$ and $CXCR5^{lo}$ subset in SLE patients as compared to healthy control. **B,**

elevated PD-1 expression (MFI) in $CXCR5^{hi}$ and $CXCR5^{lo}$ subset in SLE patients as compared to healthy control and BD patients. Data points represent individual subjects; horizontal lines represent the mean value with standard error of the mean. Statistical analysis was performed using One-way ANOVA. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. no significance). See figure 1 for additional definitions.

Figure 3. PD-1 expression on circulating $CXCR5^{hi}$ subset correlates with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and expansion of circulating plasmablasts. **A,** No association between percentages of activated $CXCR5^{hi}$ subset in circulating $CD4^+$ T cells and SLEDAI (Spearman r = - 0.21, p = 0.28) in systemic lupus erythematosus (SLE) patients with active disease. **B,** Significant correlation between PD-1 MFI in circulating $CXCR5^{hi}$ subset and clinical activity as measured by SLEDAI (Spearman r = 0.43, p = 0.03) in SLE patients with active disease. **C,** No association between ICOS MFI in $CXCR5^{hi}$ subset and SLEDAI (Spearman r = 0.16; p = 0.52) in SLE patients with active disease. **D,** expansion of circulating plasmablast ($CD19^+IgD^-CD38^+$) in SLE patients as compared to Behçet's disease (BD) and healthy control (H). Statistical analysis was performed using One-way ANOVA (* $p < 0.05$). **E,** Correlation between percentage of circulating plasmablasts and PD-1 MFI in blood activated $CXCR5^{hi}$ subset ($CD45RA^-CXCR5^{hi}$) in SLE patients (Spearman r = 034; p = 0.02; left panel). Percentage of circulating plasmablasts in the presence or absence of prednisone, antimalarial, and azathioprine treatments in the SLE patient cohort. Statistical analysis was performed using Student's t test (n.s. = no significance). Data points represent individual subjects; horizontal lines represent the mean values with standard error of the mean.

Figure 4. Functional and phenotypic analysis of circulating $CXCR5^{hi}PD\text{-}1^{hi}$ cells. **A,** Representative FACS analysis of intracellular IL-21 staining in circulating $CXCR5^{hi}PD\text{-}1^{lo}$ cells and $CXCR5^{hi}PD\text{-}1^{hi}$ cells; values represent percentages of $IL\text{-}21^{+}$ cells (left panel). Increased percentage of $IL\text{-}21^{+}$ cells in circulating $CXCR5^{hi}$-$PD\text{-}1^{hi}$ population as compared to $CXCR5^{hi}PD\text{-}1^{lo}$ population ($p = 0.02$; right panel). **B,** Representative FACS analysis of CCR7 expression in circulating naïve, $CXCR5^{hi}PD\text{-}1^{lo}$, and $CXCR5^{hi}PD\text{-}1^{hi}$ subsets; gating of $PD\text{-}1^{hi}$ and $PD\text{-}1^{lo}$ subset is based on PD-1 expression on circulating naïve subset (left panel). Significant decrease in CCR7 expression in $CXCR5^{hi}PD\text{-}1^{hi}$ cells as compared to $CXCR5^{hi}PD\text{-}1^{lo}$ cells ($p = 0.03$). CCR7 expression is represented as relative CCR7 MFI to that of circulating naïve subset (right panel). **C,** Representative histogram showing the expression of Bcl6 in SLE circulating $CXCR5^{hi}PD\text{-}1^{hi}$ cells, Tonsil naïve cells ($CD4^{+}TCR\beta^{+}CD45RA^{+}$), and Tonsil follicular helper T cells (Tfh; $CD4^{+}TCR\beta^{+}CD45RA^{-}CXCR5^{hi}PD\text{-}1^{hi}$). Statistical analysis was performed using Student's t test.

Figure 5. Model of altered follicular helper T cell phenotype in systemic lupus erythematosus. Presentation of autoantigen by dendritic cells (DC) to T cells leads to the differentiation of T helper (Th) cells and follicular helper T precursors (preTfh). Tfh-destined cells migrate to border of T-cell zones and B-cell follicles, where they contact autoantigen-activated B cells. Subsequent migration of both subsets deeper into the follicle leads to germinal center (GC) formation. GC Tfh cells are notable for high CXCR5, PD-1, and Bcl6 expression, as well as production of IL-21. In the GC, Tfh cells promote class switching and affinity selection of B cells via secretion of cytokines (eg. IL-21) and expression of cell bound molecules (eg. PD-1). The result is the production of

high-affinity autoantibody-secreting plasma cells, leading to pathology and disease activity. In parallel, there is an expansion of Tfh-like cells in the peripheral blood. Akin to Tfh cells, these Tfh-like cells also express high levels of CXCR5 and PD-1, and are enriched with IL-21 producers. High PD-1 expression in blood Tfh-like cells is associated with disease activity, plasmablast expansion, and autoantibody production. Unlike GC Tfh cells, circulating Tfh-like cells have low levels of Bcl6, indicating that they may have relocated to the periphery pre- or post-germinal center reaction. Importantly, these circulating Tfh-like cells express low levels of CCR7, which differentiate them from previously described central memory T cells with high level of CXCR5.

TABLES

	SLE patients (n = 49)	Behçet's disease patients (n = 28)	Healthy Controls (n = 16)
Demographics			
Female, no. (%)	46 (94)	22 (79)	12 (75)
Age, years	37 (30-48)	48 (40-56)	28 (27-32)
Race, no. (%)			
White	32 (65)	20 (71)	13 (81)
Non-white	17 (35)	8 (29)	3 (19)
SLE manifestations			
Disease duration, years	11 (5-16)	--	--
SLEDAI			
0 – 4, no. (%)	38 (77%)	--	--
5 – 9, no. (%)	5 (10%)	--	--
> 9, no. (%)	6 (12%)	--	--
Clinical and laboratory features, %			
Skin disease	16	--	--
Arthritis	8	--	--
Serositis	0	--	--
CNS disease	0	--	--
Renal manifestation	27	--	--
Low Complements	12	--	--
Anti-dsDNA	14	--	--
Medication usage, %			
Steroids	69	--	--
Antimalarials	45	--	--
Cytotoxic agents	22	--	--

* Except where indicated otherwise, values are the median (interquartile range)

Table 1. Study population characteristics.