ARTHRITIS & RHEUMATISM
Vol. 62, No. 1, January 2010, pp 234–244
DOI 10.1002/art.25032

Expansion of Circulating T Cells Resembling Follicular Helper T Cells Is a Fixed Phenotype That Identifies a Subset of Severe Systemic Lupus Erythematosus

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Objective. **In the sanroque mouse model of lupus, pathologic germinal centers (GCs) arise due to increased numbers of follicular helper T (Tfh) cells, resulting in high-affinity anti–double-stranded DNA antibodies that cause end-organ inflammation, such as glomerulonephritis. The purpose of this study was to examine the hypothesis that this pathway could account for a subset of patients with systemic lupus erythematosus (SLE).**

Methods. **An expansion of Tfh cells is a causal, and therefore consistent, component of the sanroque mouse phenotype. We validated the enumeration of circulating T cells resembling Tfh cells as a biomarker of this expansion in sanroque mice, and we performed a comprehensive comparison of the surface phenotype of circulating and tonsillar Tfh cells in humans. This circulating biomarker was enumerated in SLE patients (n = 46), Sjögren's syndrome patients (n = 17), and healthy controls (n = 48) and was correlated with disease activity and end-organ involvement.**

Results. **In sanroque mice, circulating Tfh cells increased in proportion to their GC counterparts, making circulating Tfh cells a feasible human biomarker of this novel mechanism of breakdown in GC tolerance. In a subset of SLE patients (14 of 46), but in none of the controls, the levels of circulating Tfh cells (defined as circulating CXCR5+CD4+ cells with high expression of Tfh-associated molecules, such as inducible T cell costimulator or programmed death 1) were increased. This cellular phenotype did not vary with time, disease activity, or treatment, but it did correlate with the diversity and titers of autoantibodies and with the severity of end-organ involvement.**

Conclusion. **These findings in SLE patients are consistent with the autoimmune mechanism in sanroque mice and identify Tfh effector molecules as possible therapeutic targets in a recognizable subset of patients with SLE.**

The clinical phenotype of systemic lupus erythematosus (SLE) is pleomorphic (1–3). One explanation for this is an underlying heterogeneity in the cellular pathways that become dysregulated to cause the disease (4). The long-term outcome and the response to treatment are also variable. The selection of therapeutic agents for the treatment of SLE is largely based on patterns of end-organ manifestations, which are likely to be remote from the fundamental pathophysiologic defects. Identification of cellular subsets or biomarkers that differentiate distinct lupus syndromes according to these pathophysiologic defects is a necessary first step toward

Supported by the National Health and Medical Research Council of Australia (project grants 316914 and 427619; program grant 427620).

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Submitted for publication September 12, 2008; accepted in revised form September 8, 2009.

more-accurate prognoses, better therapeutic decisions, and ultimately, more precise treatment strategies.

Production of autoantibodies is a consistent component of the lupus phenotype. Illuminating the defects that result in the production of these autoantibodies would be expected to be informative for our understanding of the mechanism of disease, since they can be detected well before the clinical manifestations of lupus appear (5), and in some cases, they are pathogenic.

Several lines of evidence point to defects in the selection of germinal center (GC) B cells in the production of autoantibodies in lupus patients. First, these autoantibodies are somatically mutated high-affinity IgG (6). Second, GCs are abundant in secondary lymphoid organs in mouse models of SLE (7), and there is evidence that GCs are overactive in human lupus (8,9). Third, lupus autoantigens are predominantly intracellular antigens, which are selectively extruded on cell surface blebs during apoptosis (suggesting that aberrant disposal of apoptotic cells may contribute to SLE) (10). Uniquely high rates of apoptosis occur within GCs, accounting for the large numbers of tingible body macrophages that characterize these lymphoid regions. In some SLE patients, there may be a reduction in the number of such macrophages (11). Fourth, some immunoglobulin genes from autoreactive memory B cells contain somatic mutations (12,13). Finally, self-reactive B cells bearing V_H4.34 antibodies against *N*-acetyllactosamine determinants of i/I blood group antigen and CD45 are selectively excluded or eliminated from GCs in normal tonsils but become frequent in tonsillar GCs from patients with SLE (14).

Tolerance mechanisms acting during antibody selection within GCs are not well understood, and dysregulated GC selection could arise by a range of possible pathways (15). We identified 1 pathway whose dysregulation produces a florid lupus phenotype in sanroque mice, which was discovered by surveying mice segregating *N*-ethyl-*N*-nitrosourea–induced gene variants for the expression of antinuclear autoantibodies (16). The sanroque mouse phenotype results from homozygous missense mutations in a ubiquitin ligase family member, roquin (*Rc3h1*). Furthermore, we have shown that the previously reported expansion of follicular helper T (Tfh) cells is causally related to the abundant GCs that form in the absence of foreign antigen, to the production of high-affinity anti–double-stranded DNA (anti-dsDNA) antibodies, and to end-organ disease (17).

Normally, during T cell–dependent antibody responses to antigen, affinity maturation depends on the selection by Tfh cells of GC B cells that express antibodies that bind the antigen with high affinity, while those B cells that either lose affinity for antigen or become self-reactive fail to elicit Tfh help and undergo apoptosis (18,19). The dysregulated accumulation of Tfh cells in sanroque mice highlights the importance of controlling this pathway to prevent the formation of dsDNA autoantibodies in lupus and raises the possibility that dysregulated Tfh cell activity may contribute to SLE in humans.

Since Tfh cells are expanded in sanroque mice, we sought evidence for this phenotype in human lupus. We identified a subset of SLE patients in whom circulating CXCR5+CD4+ T cells with high expression of inducible T cell costimulator (ICOS) and/or programmed death 1 (PD-1) are increased. Since these cells share other phenotype characteristics of Tfh cells in secondary lymphoid organs, we refer to them as circulating Tfh cells. An increase in circulating Tfh cells appears to be a stable phenotype rather than a marker of disease activity. Taken together, our findings provide evidence of a cellular phenotype consistent with defects identified in the sanroque mouse.

PATIENTS AND METHODS

Study subjects. A total of 46 SLE patients and 31 healthy control subjects were recruited as part of the Australian Point Mutation in Systemic Lupus Erythematosus (APOSLE) study. An additional 20 lupus patients were recruited via Imperial College London. All lupus patients met the American College of Rheumatology criteria for the classification of SLE (3). A total of 17 patients with Sjögren's syndrome (SS) according to the American–European Consensus Group Criteria for SS (20), and 17 control subjects were recruited in Canberra. Tonsils and blood samples were obtained from children without autoimmune disease who were undergoing elective tonsillectomies. Ethics approval was obtained from each jurisdictional ethics committee.

Mice. Roquin$^{san/san}$ mice were maintained on a C57BL/6 background and, together with wild-type C57BL/6 mice, were housed under specific pathogen–free conditions. All animal procedures were approved by the Australian National University Animal Ethics and Experimentation Committee.

Antibodies and reagents. All antibodies and conjugates were from BD PharMingen (San Diego, CA) except biotinylated ICOS and biotinylated mouse IgG1κ isotype control (both from eBioscience, San Diego, CA), streptavidin Qdot 605 (Invitrogen, San Diego, CA), and 7-aminoactinomycin (7-AAD; Molecular Probes, Eugene, OR).

Cell isolation and flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density-gradient centrifugation on Ficoll-Paque Plus (Amersham Biosciences, Little Chalfont, UK). Single-cell suspensions of mouse cells were prepared from peripheral blood, spleens, and pooled peripheral lymph nodes obtained from

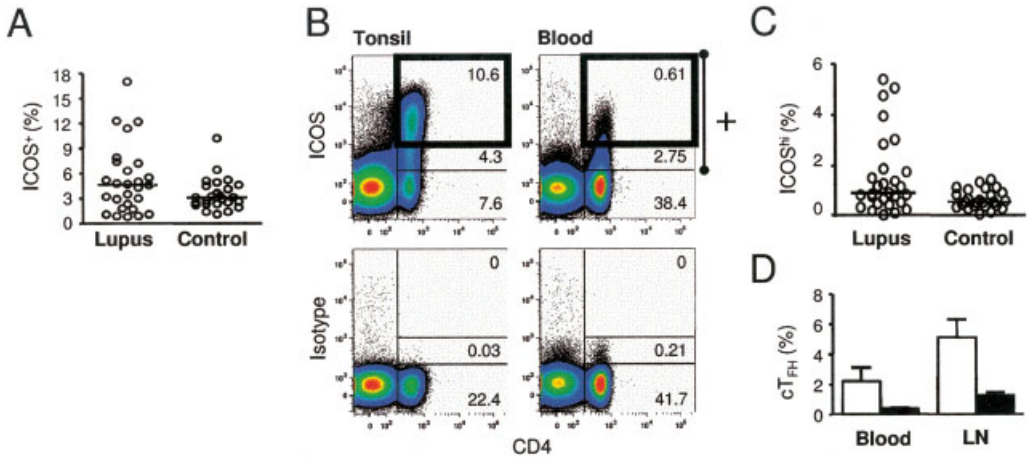

Figure 1. Inducible T cell costimulator (ICOS) expression in CD4+ cells. **A,** Percentage of ICOS+CD4+ lymphocytes in 29 systemic lupus erythematosus (SLE) patients and 25 age-matched healthy controls. Each data point represents an individual subject; horizontal lines show the median. **B,** Representative expression of ICOS and isotype control versus CD4 expression by flow cytometry, gated on viable (7-aminoactinomycin–negative [7-AAD–]) lymphocytes from tonsil and blood (same donor). Values in each CD4+ gate ($ICOS^{high}$ [black box], ICOS-positive [$ICOS^{intermediate}$ plus $ICOS^{high}$] [+], and $ICOS^{negative}$) are the percentage of viable lymphocytes. The gate for positive cells allows 0.5% positive isotype control staining in blood CD4+ cells. **C,** Percentage of $ICOS^{high}CD4+$ lymphocytes in SLE patients and controls. Each data point represents an individual subject; horizontal lines show the median. **D,** Comparison of PD-1+CXCR5+ circulating follicular helper T cells (cT_{FH}) in blood and lymph nodes (LN) from sanroque mice (open bars) and wild-type mice (solid bars). Values are the mean and SD of 3 mice per group.

nonimmunized mice. For fluorescence-activated cell sorter analysis, labeled cells were collected by multiparameter flow cytometry (LSR II flow cytometer; Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Complement levels were measured by immunoturbidimetry (Abbott, Abbott Park, IL). Serum anti-dsDNA antibodies were assessed by *Crithidia luciliae* immunofluorescence (Immuno Concepts, Sacramento, CA), enzyme-linked immunosorbent assay (ELISA; Orgentec Diagnostika, Mainz, Germany), and multiaddressable bead array (Fidis Connective 10 kit; BMD, Marne-la-Vallée, France). Absolute lymphocyte counts were obtained by automated complete blood cell counts.

Real-time polymerase chain reaction (PCR). Tonsil or PBMCs were sorted using a FACSAria (BD Biosciences, San Jose, CA) into CD4+ subgroups based on their expression of ICOS and CXCR5. RNA was extracted from sorted cells using the *mir*Vana microRNA isolation kit (Ambion, Austin, TX), and real-time PCR was performed as described previously (21) using the following primers: for Bcl-6, 5′-AAGGCCAGTGAAGCAGAGA-3′ and 5′-CCGATAGGCCATGATGTCT-3′; and for interleukin-21 (IL-21), 5′-CACAGACTAACATGCCCTTCAT-3′ and 5′-GAATCTTCACTTCCGTGTGTTCT-3′.

Serum analysis. IL-21 was measured by ELISA using anti-human IL-21 clone J148-1134 (BD Biosciences) and biotinylated mouse (IgG1κ) anti-human IL-21 clone I76-539 (BD Biosciences). Total serum IgG was determined by immunoturbidimetry (Architect c8000 analyzer; Abbott).

Statistical analysis. Data were analyzed with Prism software (version 4.0c for Macintosh; GraphPad Software, San Diego, CA), using the Mann-Whitney test or the Kruskal-Wallis test (with Dunn's post hoc test) and Fisher's exact test for the statistical significance of differences in proportions (unpaired). For correlation analyses, either Spearman's r or an r^2 value derived from Pearson's r was calculated, and the significance was evaluated using the t statistic.

RESULTS

Discrete $ICOS^{high}CD4+$ T cell population in the blood of a subset of SLE patients. An increased fraction of ICOS+CD4+ lymphocytes has been reported in lupus patients. The size of this fraction varies significantly in different cohorts as well as according to how the cell population is defined (22–24). Overall, this finding is consistent with abnormal T cell activation in SLE, but this has not been shown to discriminate different subsets of lupus patients. We determined the fraction of ICOS+CD4+ T cells (using isotype control staining for each subject to set the ICOS+ threshold) and identified a modest difference between 29 SLE patients and 25 age-matched healthy controls (median 4.6% versus 3.1%; $P = 0.14$) (Figure 1A).

In addition to expression on activated T cells, ICOS is expressed on Tfh cells in secondary lymphoid organs. Indeed, the magnitude of ICOS expression

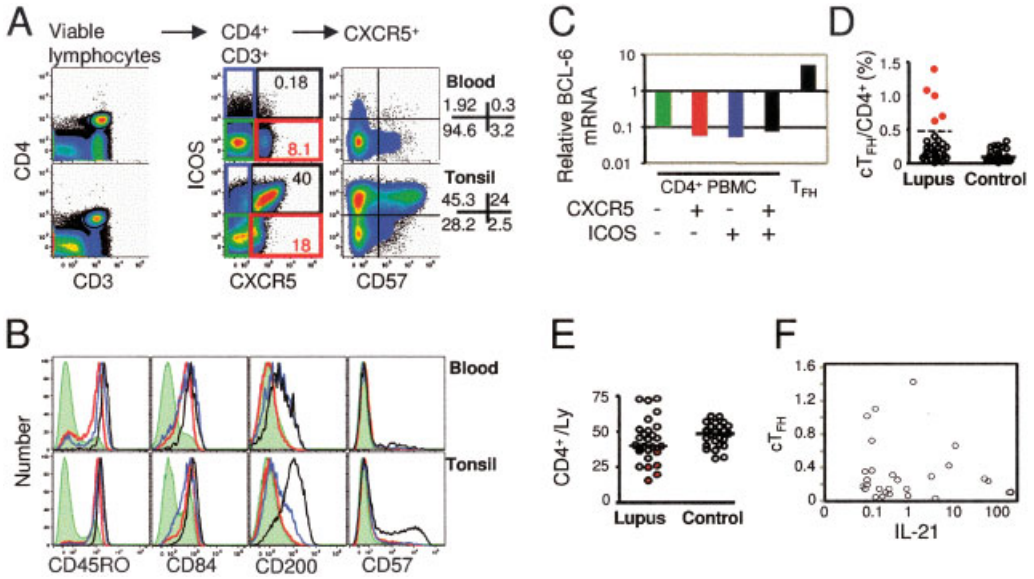

Figure 2. Circulating and tonsillar follicular helper T (Tfh) cells. **A,** Representative fluorescence-activated cell sorter (FACS) analysis of tonsil and blood cells, gated on viable (7-aminoactinomycin–negative [7-AAD–]) lymphocytes, plus CD3+CD4+ cells, plus CXCR5+ cells. Values in each compartment are the percentage of viable lymphocytes. **B,** Representative histograms showing the expression of CD45RO, CD84, CD200, and CD57 on tonsil and blood CD4+CD3+ lymphocytes from the experiment shown in **A**. CXCR5+ICOS^{high} (circulating Tfh cells) and Tfh cells (black) are shown. Overlays show subsets gated on CXCR5–ICOS^{high} (blue), CXCR5+ but not ICOS^{high} (red), and CXCR5– but not ICOS^{high} (green shaded histograms). **C,** Relative abundance of Bcl-6 mRNA in peripheral T cell subsets and Tfh cells from tonsil. **D,** Summary of FACS analysis of peripheral blood from 29 systemic lupus erythematosus (SLE) patients and 25 controls. Circulating Tfh cells are expressed as a percentage of CD4+7-AAD– lymphocytes. Each data point represents an individual subject; horizontal broken line at 0.48% indicates the threshold of 4 SD above the control mean for defining a high level of circulating Tfh cells. Solid red circles indicate SLE patients with high levels of circulating Tfh cells; black circles indicate the other SLE patients. **E,** CD4+ T cells as a percentage of lymphocytes (Ly) in SLE patients and healthy controls. Each data point represents an individual subject; horizontal lines show the median. **F,** Correlation of serum interleukin-21 (IL-21) levels with expression of circulating Tfh cells in SLE patients. ICOS = inducible T cell costimulator; PBMC = peripheral blood mononuclear cell; cT_{FH} = circulating follicular helper T cells.

distinguishes activated T cells and Tfh cells (25). Analysis of circulating CD4+ T cells using an ICOS threshold more specific for tonsillar Tfh cells (Figures 1B and C) identified a significant difference between SLE patients and controls (median percentage of ICOS^{high}CD4+ lymphocytes 0.93% versus 0.58%; $P = 0.02$) (Figure 1C). This difference was accounted for by a 5–9-fold increase in ICOS^{high}CD4+ cells in 6 of the 29 SLE patients, whereas in the remainder of the patients, the number of ICOS^{high}CD4+ T cells was similar to that in the controls.

Similarity of phenotype of circulating ICOS^{high} CXCR5+CD4+ T cells and tonsillar Tfh cells. In sanroque mice, abundant GCs and overproduction of Tfh cells led to aberrant selection of high-affinity self-reactive B cells and features of lupus (17). We enumerated Tfh cells in lymph nodes and peripheral blood from a group of sanroque mice and found a significant expansion in both compartments as compared with their wild-type littermates (Figure 1D). Based on this finding, we postulated that an increase in circulating cells derived from GC Tfh cells, rather than simply an increase in activated T cells, could account for the increased fraction of circulating ICOS^{high}CD4+ cells in a subset of patients with SLE.

Functional studies have demonstrated that Tfh cells are ICOS^{high}CXCR5^{high} (26). To increase the level of discrimination between circulating Tfh cells and activated T cells, we set a threshold for ICOS that was at least 2 times higher than the fluorescence intensity for defining ICOS+ cells and was equivalent to the threshold used to identify Tfh cells in tonsils (i.e., circulating Tfh cells were ICOS^{high}CXCR5+CD4+) (Figure 2A). To validate this definition, we compared the surface

phenotype of CD4+ T cell subsets defined by ICOS and CXCR5 expression in the blood and tonsils of 18 healthy children (Figures 2A and B).

Circulating ICOS^{high}CXCR5+CD4+ cells also expressed high levels of CD45RO and CD84, comparable to the levels on Tfh cells (Figure 2B). This pattern was uniform across samples. In tonsillar CD4+ T cells, CD200 has been shown to be specific for the Tfh cell subset (27). Similarly, in blood samples, we found a covariation of CD200 and ICOS expression on CD4+ T cells (Figure 2B). CD57 identified a subset of Tfh cells (Figures 2A and B), although the capacity to provide B cell help by ICOS^{high}CXCR5^{high} cells appears to be independent of CD57 expression (26). While CD57 expression on circulating CD4+ cells was lower than that on tonsillar T cells, circulating ICOS^{high}CXCR5+CD4+ T cells contained a higher fraction of CD57+ cells than did the other circulating cell subsets (Figure 2B). Circulating Tfh cells also expressed CD10 (data not shown). This comprehensive surface phenotype is consistent with the postulate that ICOS^{high}CXCR5+CD4+ cells in blood are related to Tfh cells (28).

Bcl-6 is a transcriptional repressor expressed in GC B cells and at least a subset of Tfh cells within GCs (29,30); however, we found similar levels of Bcl-6 expression in circulating Tfh cells and other T cell subsets (Figure 2C). Similarly, IL-21 messenger RNA expression was very low in all circulating CD4+ cells sorted ex vivo. Serum IL-21 was increased in a significant subset of lupus patients, but serum IL-21 levels did not vary with the levels of circulating Tfh cells (Figure 2F).

Identification of a subset of SLE patients with an increased proportion of circulating Tfh cells in the CD4+ T cell compartment. Reanalysis of circulating Tfh cells defined as ICOS^{high}CXCR5+CD4+ in SLE patients and controls yielded results similar to those from the analysis for ICOS^{high}CD4+ T cells. The circulating Tfh cell fraction was increased in SLE patients as compared with controls (0.21% versus 0.09%; $P = 0.02$) (Figure 2D). In contrast, median levels of the CXCR5+CD4+ fraction were similar in the 2 groups (10.2% and 10.3%, respectively; $P = 0.94$) (data not shown). The difference in circulating Tfh cells as a proportion of CD4+ T cells in the SLE patient and age-matched healthy control groups was still accounted for by a subset of 5 SLE patients of the 29 patients tested (17.2%) in whom the proportion of circulating Tfh cells was >4 SD above the mean value in the controls (Figure 2D). Analysis according to CD57+CXCR5+CD4+ T cells yielded the same results (data not shown). Furthermore, this subset of SLE patients with a high proportion of circulating Tfh cells had a higher proportion of CD57+ICOS^{high} cells among their CXCR5+CD4+ lymphocytes (median 0.51%) than did the remaining 24 patients (0.08%; $P < 0.01$) and the controls ($P < 0.001$), who had a low proportion of circulating Tfh cells. All SLE patients with circulating Tfh^{high} cells had CD4+ T cell lymphopenia (Figure 2E). In 4 of 5, the lymphocyte fraction was >2 SD below the mean of 47.3% in 25 control subjects (this was also the case for 1 patient with active SLE receiving cyclophosphamide who had low levels of circulating Tfh cells).

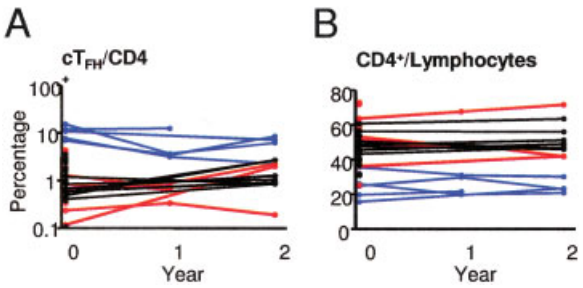

Figure 3. Stability of the circulating follicular helper T (Tfh) cell phenotype over time in systemic lupus erythematosus (SLE) patients and healthy controls. **A,** ICOS^{high}CXCR5+ Tfh cells as a percentage of CD4+7-aminoactinomycin–negative lymphocytes, normalized against the median value in healthy controls at each time point. **B,** CD4+ cells as a percentage of lymphocytes in the same SLE patients and controls. Lines indicate subjects with data from >1 time point. Black lines and symbols indicate healthy controls, blue lines and symbols indicate SLE patients with high levels of circulating Tfh cells, and red lines and symbols indicate SLE patients with low levels of circulating Tfh cells.

Stability of the circulating Tfh^{high} phenotype over time. To determine whether the circulating Tfh^{high} phenotype is a marker of disease activity or whether it is a fixed phenotype and is therefore likely to be related to fundamental pathogenesis, we performed followup analyses over 26 months in subsets of patients with high and low circulating levels of Tfh cells (as defined in Figure 2D). The between-group differences in the number of circulating Tfh cells seen at baseline persisted over this interval (Figure 3). Only 1 patient initially classified as having high levels of circulating Tfh cells showed a change in classification; this occurred at the 26-month time point, when this patient's circulating levels of Tfh cells fell to just within the range of values seen in patients and controls with low levels of circulating Tfh cells.

None of the subjects who were initially classified as having low levels of circulating Tfh cells developed a high fraction of ICOS^{high}CXCR5+ T cells. Levels of CD57+CXCR5+ cells were also stable over the 26-

month period (data not shown). These data suggest that an increase in circulating Tfh cells is a relatively fixed phenotype in a subset of lupus patients.

To exclude secondary changes in the proportions of circulating Tfh cells due to changes in other subsets, we also confirmed that the absolute number of circulating Tfh cells (expressed as cells/ml) was increased. At the 26-month time point, the median number of circulating Tfh cells in the patients with low levels of circulating Tfh cells was 884 cells/ml (range 542–1,587), as compared with 3,661 cells/ml in those with high levels of circulating Tfh cells (range 1,282–4,432). Thus, despite substantial CD4+ cell lymphopenia, SLE patients with a high proportion of circulating Tfh cells also had a relatively high absolute number of circulating Tfh cells.

Stability of the circulating Tfh^{high} phenotype in relation to disease activity. To determine whether the circulating Tfh cell phenotype might identify a pathogenically distinct subset of patients with lupus or whether it simply reflects disease activity, we compared clinical and serologic evidence of disease activity at the time the circulating Tfh cell phenotype was initially studied. There was no correlation between the fraction of circulating Tfh cells in the blood and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score (31) or the use of immunosuppressive drugs. In the 5 SLE patients with high levels of circulating Tfh cells (ranked highest to lowest according to the proportion of circulating Tfh cells), the SLEDAI scores reflecting disease severity at the time of scoring were 0, 2, 2, 8, and 14. At the time of determination of circulating Tfh cells, 3 of these 5 patients were not receiving immunosuppressive medication, 1 was taking mycophenolate mofetil and was undergoing tapering of the prednisolone (8 mg/day), and 1 was taking prednisolone (20 mg/day), azathioprine, and hydroxychloroquine.

In the group of patients with low levels of circulating Tfh cells (n = 22 with available data), the SLEDAI scores ranged from 0 to 18, with a score of ≥10 in 2 of them. Four of the 22 patients with low levels of circulating Tfh cells were taking no immunosuppressive agents, and 12 were taking medications other than hydroxychloroquine (including 2 who were receiving pulse cyclophosphamide and 1 who was taking methotrexate). In addition, there were no statistically significant differences in the levels of C3 or C4 between the patients with high levels of circulating Tfh cells, the patients with low levels of circulating Tfh cells, and the healthy control groups (median C3 levels 1.74, 1.50, and 1.58 gm/liter, respectively [$P = 0.43$ by Kruskal-Wallis test], and median C4 levels 0.18, 0.26, and 0.27 gm/liter, respectively [$P = 0.26$]). While 4 of the 5 patients with high levels of circulating Tfh cells had high levels of antichromatin antibodies at some time during their disease course, patients with high levels of anti-dsDNA antibodies at the time circulating Tfh cells were tested were not overrepresented in the group with high levels of circulating Tfh cells.

Overall, these data suggest that the circulating Tfh^{high} phenotype is not a function of current disease activity or treatment. Instead, they suggest that patients with high levels of circulating Tfh cells may, over the course of their illness, be more likely than other SLE patients to experience severe manifestations of disease. The median age of the SLE patients in the groups with high versus low levels of circulating Tfh cells was similar (39.2 years versus 42.7 years; $P = 0.88$).

Expression of PD-1 on circulating Tfh cells. PD-1 is expressed at high levels on mouse Tfh cells (32). During the course of our study, a new monoclonal antibody for human PD-1 became available, and we found this to be both sensitive and specific for human Tfh cells (33). In human tonsils, PD-1 expression on CD4+ T cells varied from almost zero to high levels and varied in parallel with the expression of ICOS and CXCR5 (Figures 4A and B). The highest levels of PD-1 expression in tonsillar CD4+ cells were in the CXCR5^{high} subset (i.e., Tfh cells), whereas a significant proportion of ICOS+CD4+ cells were found in the CXCR5– population (Figure 4B). CD57+ T cells were found within the PD-1+ subset. Coexpression of CD4 and high levels of PD-1 appeared to identify Tfh cells.

In the 25 subjects whose PBMCs were analyzed for both ICOS and PD-1 (6 healthy control subjects, 6 lupus patients, and 13 SS patients), there was a strong correlation between the expression of these 2 molecules on circulating CD4+ cells (Spearman's r = 0.69). In those with staining of CXCR5+CD4+ T cells for both ICOS and PD-1, there was a strong correlation between circulating Tfh cells as defined by the expression of ICOS and CXCR5 and circulating Tfh cells as defined by the expression of PD-1 and CXCR5 (r = 0.76) (Figure 4C). An equally robust correlation was observed between ICOS^{high}CXCR5+CD4+ T cells and PD-1^{high}CD4+ T cells (data not shown), making PD-1 alone on circulating CD4+ T cells a biomarker of Tfh cells.

Analysis of a second group of lupus patients (n = 17) and healthy controls (n = 6) for PD-1^{high} CXCR5+CD4+ lymphocytes revealed an increased fraction in the lupus cohort (median 1.16% versus 0.33%; $P = 0.0046$) (Figure 4D). A similar result was obtained when circulating Tfh cells were defined as

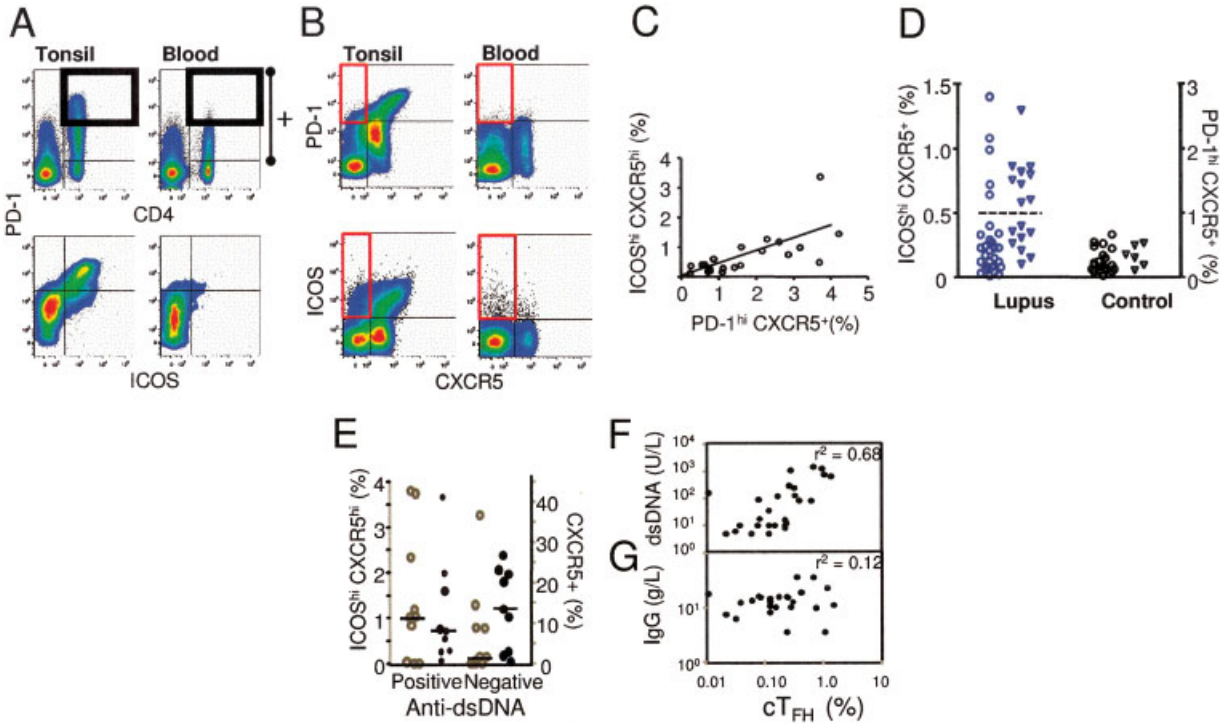

Figure 4. Programmed death 1 (PD-1) expression by follicular helper T (Tfh) cells and circulating Tfh cells. **A,** Fluorescence-activated cell sorter analysis of PD-1 versus CD4 and inducible T cell costimulator (ICOS), gated on viable lymphocytes derived from tonsil and blood, to show the PD-1^{intermediate} (<0.5% in CD4+ control peripheral blood mononuclear cells [PBMCs]) and PD-1^{high} cells (black box). All PD-1+ cells are indicated (+). **B,** Comparison of PD-1 and ICOS expression according to the expression of CXCR5 on CD4+CD3+7-aminoactinomycin–negative tonsil and blood cells. Samples are from the same subject as in **A**. **C,** Scatterplot of the percentage of PD-1^{high}CXCR5+ cells versus ICOS^{high}CXCR5+ cells among CD4+CD3+ PBMCs from 6 controls, 6 systemic lupus erythematosus (SLE) patients, and 13 Sjögren's syndrome patients, showing a strong correlation (r = 0.76, $P < 0.0001$). **D,** Summary of circulating Tfh cell expression profiles as defined by ICOS or PD-1 expression in SLE patients and healthy controls. Each data point represents an individual subject; horizontal broken line indicates the threshold for defining the circulating Tfh^{high} subset, which is 4 SD above the mean in healthy controls. On the left y-axis, circles indicate ICOS^{high}CXCR5+ cells among the CD4+ lymphocytes; on the right y-axis, triangles indicate PD-1^{high}CXCR5+ cells among the CD4+ lymphocytes. **E,** Identification of SLE patients with anti–double-stranded DNA (anti-dsDNA) antibodies according to an increased percentage of circulating Tfh cells, as determined in an independent cohort of patients. On the left y-axis, open circles indicate CXCR5^{high}ICOS^{high}circulating Tfh cells; on the right y-axis, solid circles indicate CXCR5+CD4+ T cells. Horizontal lines show the median. **F,** Relationship between the percentage of circulating Tfh cells and the titer of dsDNA antibodies (r^2 = 0.68, $P < 0.0001$). **G,** Relationship between the percentage of circulating Tfh cells and the total serum IgG level (r^2 = 0.12). cT_{FH} = circulating follicular helper T cells.

PD-1^{high} alone. Notably, PD-1 expression was increased overall in CD4+ cells from SLE patients, but as with ICOS, there was no difference in the percentage of CD4+ cells expressing CXCR5 in SLE and control subjects (12.7% versus 11.0%; $P = 0.42$) (data not shown).

Increased autoantibody formation and end-organ damage in SLE patients with high levels of circulating Tfh cells. Since a substantial increase in circulating Tfh cells was found in only a subset of our lupus cohort, we were interested to know whether this cellular phenotype identified patients with different clinical manifestations of lupus. From data collected in a questionnaire completed by treating physicians who were blinded to the results of the cellular phenotyping (PAG, DAF, and MCC), we compared patients with high versus low levels of circulating Tfh cells for a history of clinical and laboratory manifestations of lupus. Identifying the subset with high levels of circulating Tfh cells as those with values >4 SD above the mean in controls (n = 14) (Figure 4D) revealed associations with multiple high-titer autoantibodies, as well as antibody-mediated end-organ inflammation. A similar trend was observed in a further independent cohort (Figure 4E).

Compared with low levels of circulating Tfh cells, increased numbers of circulating Tfh cells were associated with glomerulonephritis on kidney biopsy (7 of 14 versus 4 of 32; $P = 0.01$), thromboembolic disease (deep venous thrombosis or pulmonary embolism; 6 of 14

Table 1. Correlation between clinical manifestations and the presence of Tfh cell–associated molecules on circulating CD4+ cells in patients with SLE*

SLE manifestation	Circulating Tfh cells†		
	High expression	Low expression	P‡
Photosensitivity, acute or subacute cutaneous lupus	7/14 (50)	27/32 (84)	0.03
Arthritis	10/14 (71)	28/32 (88)	0.22
Serositis	5/14 (36)	9/32 (28)	0.73
Kidney damage§	9/14 (64)	6/32 (19)	0.0051
Glomerulonephritis noted on biopsy	7/14 (50)	4/32 (13)	0.01
Seizures	3/14 (21)	8/32 (25)	1.0
Vasculitis of the fingers and/or toes	3/14 (21)	3/32 (9)	0.35
Thrombocytopenia	4/14 (29)	1/32 (3)	0.025
Thromboembolic disease			
Deep venous thrombosis or pulmonary embolism	6/14 (43)	4/32 (13)	0.047
Autoantibodies			
High titers of anti-dsDNA antibody	7/11 (64)	5/27 (19)	0.02
Antibody against any extractable nuclear antigen	12/13 (92)	13/28 (46)	0.0061
Anti-SSA antibody	10/13 (77)	10/27 (37)	0.04
High titers of anticardiolipin antibody	5/12 (42)	1/29 (3)	0.0053
Antinuclear antibody	13/13 (100)	30/30 (100)	–

* Values are the number of patients positive/total number tested (%). Anti-dsDNA = anti–double-stranded DNA.
† The group with high expression of follicular helper T (Tfh) cells was defined according to the percentage of circulating CXCR5+CD4+ T cells with high levels of expression of inducible T cell costimulator (systemic lupus erythematosus [SLE] group 1; n = 29) or programmed death 1 (SLE group 2; n = 17). The group with low expression was defined as all other SLE patients (n = 32).
‡ *P* values less than 0.05 were considered significant, and those less than 0.005 were considered highly statistically significant, as determined by Fisher's exact test.
§ Defined as either biopsy-proven glomerulonephritis, urinary red cell casts, proteinuria of >0.5 gm/day, or a glomerular filtration rate of <60 ml/minute.

versus 4 of 32 [$P = 0.047$]), thrombocytopenia (4 of 14 versus 1 of 32; $P = 0.025$), and high titers of autoantibodies (for anti-dsDNA antibodies, 7 of 11 versus 5 of 27 [$P = 0.02$]; for anticardiolipin antibodies, 5 of 12 versus 1 of 29 [$P = 0.0053$]; and for antibodies to extractable nuclear antigens, 12 of 13 versus 13 of 28 [$P = 0.0061$]) (Table 1). Indeed, there was a strong correlation between the titer of dsDNA antibodies and the percentage of circulating Tfh cells ($r^2 = 0.68$, $P < 0.0001$) (Figure 4F), whereas there was no correlation with the total serum IgG concentration (Figure 4G).

Other manifestations of lupus, such as arthritis, serositis, and seizures, did not differ in frequency between the 2 groups. Consistent with our conclusion that either definition of circulating Tfh cells (i.e., CXCR5+, ICOS^{high}, or PD-1^{high}) identifies the same subset of cells, clinical associations were apparent when either cellular classification was used but not when subjects were categorized according to CXCR5+CD4+ cells (data not shown).

Increased fraction of circulating Tfh cells in a subset of SS patients. SSA and/or SSB antibodies, which typically occur in patients with SS, were seen frequently in the SLE patients with high levels of circulating Tfh cells (77% versus 37% in those with low levels of circulating Tfh cells; $P = 0.04$). Ectopic GC formation in nonlymphoid tissue is a feature of SS (34). In addition,

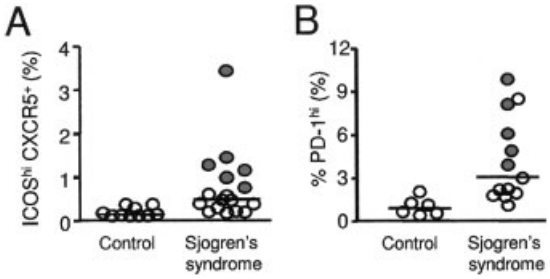

Figure 5. Circulating follicular helper T (Tfh) cells in patients with Sjögren's syndrome (SS). Summary of fluorescence-activated cell sorter analysis of peripheral blood obtained from SS patients (n = 17) and healthy controls (n = 10). **A,** Percentage of ICOS^{high}CXCR5+ circulating Tfh cells among circulating CD4+CD3+ lymphocytes. **B,** Percentage of PD-1^{high} cells among circulating CD4+CD3+ lymphocytes. Each data point represents an individual subject; horizontal lines show the median. Red circles in **B** represent the same ICOS^{high} SS patients as in **A**.

SS is typically associated with hypergammaglobulinemia, which is a consistent feature of the sanroque phenotype. We examined a cohort of 17 SS patients and found that 6 of them had a high fraction of circulating Tfh cells, whether these were defined as ICOS^{high}CXCR5+CD4+ (Figure 5A) or as PD-1^{high}CD4+ (Figure 5B). This finding supports the view that high levels of expression of PD-1 alone in CD4+ T cells may be sufficient as a biomarker of GC Tfh cell dysregulation. The absolute numbers of circulating Tfh cells in SS and SLE patients with high levels of circulating Tfh cells were similar, with a median of 3,873 cells/ml and 3,661 cells/ml, respectively. The median absolute numbers of circulating Tfh cells in SS and SLE patients with low levels of circulating Tfh cells were 2,149 cells/ml and 884 cells/ml, respectively, with 1,622 cells/ml in the healthy control group.

DISCUSSION

The clinical manifestations of SLE are diverse, and vary considerably from patient to patient, but SLE is always characterized by the production of autoantibodies. Sequence analysis of these autoantibodies indicates affinity maturation (6). Together with other evidence, this implicates immunologic tolerance mechanisms operating within GCs as candidates for the location of lupus-associated defects (7–9,17). A novel pathway that regulates tolerance in GCs was identified by analysis of sanroque mice (16). The GC tolerance pathway elucidated in sanroque mice involves regulation of B cell help provided by Tfh cells (16,17). A pathologic abundance of Tfh cells results in excessive GC formation, high levels of autoantibodies, and end-organ damage typical of lupus. Here, we provide evidence consistent with a defect in this pathway of GC Tfh cell regulation in a subset of patients with SLE.

Using a stringent cutoff level of 4 SD above the mean value in healthy control subjects, 14 of 46 SLE patients (and none of the healthy controls) were found to have an increase in circulating counterparts of Tfh cells. Compared with the remainder of the lupus patient cohort, those with high levels of circulating Tfh cells had a larger spectrum and higher titers of lupus-associated autoantibodies, were CD4+ T cell lymphopenic, and exhibited end-organ manifestations of glomerulonephritis and hematologic cytopenias, which are Tfh cell–dependent features of the sanroque mouse phenotype (17). We found no evidence to suggest that the circulating Tfh^{high} phenotype was related to age, disease activity, or immunosuppression at the time of testing. Rather, the stability of this cellular phenotype over more than 2 years of observation suggests that it is related to the fundamental pathogenesis of lupus in this subset of patients.

To test the relevance of Tfh cells to human SLE, we have necessarily relied largely on analyses of peripheral blood and the use of a circulating biomarker of Tfh cells. A comprehensive analysis of Tfh cells and circulating Tfh cells reveals important differences as well as similarities. Tfh cell–associated molecules, such as CD57, CD10, and CD200, are more prominent in circulating Tfh cells relative to other circulating CD4+ T cells, but the level of expression is lower than that on tonsillar Tfh cells. Bcl-6 messenger RNA was no more abundant in circulating Tfh cells than in other circulating CD4+ T cells, whereas consistent with previous reports, we showed that Bcl-6 is increased in tonsillar Tfh cells. This is consistent with the decrease in CXCR5 expression from Tfh cells to circulating Tfh cells (Figure 2A) and is similar to B cell memory development, which depends on the expression of Bcl-6 in GC B cells and then a down-regulation for memory B cell formation (35), and as we have shown in mice, the number of circulating Tfh cells is independent of serum IL-21 levels. Despite these differences, a parsimonious explanation for the ontogeny of circulating Tfh cells given this phenotype is that they are related to Tfh cells.

Analyses according to different definitions of circulating Tfh cells (ICOS, CXCR5, and PD-1, with or without CD57) yielded the same conclusion (Figure 5). The correlation between increased numbers of circulating Tfh cells and particular clinical manifestations of lupus is robust to variations in the definition of the subset, suggesting that the finding is substantial. Furthermore, this analysis of the ICOS^{high} subset provides an additional, or even alternative, explanation of "increased cellular activation" for the increase in ICOS+ cells, which has previously been described in lupus (36–42). Moreover, the subset of patients identified demonstrates a concordance of phenotype with that of the sanroque mice. Taken together, our findings suggest that rather than simply identifying an increase in activated T cells, the increase in circulating Tfh cells could reflect GC dysregulation that is causing the production of the high-affinity autoantibodies that characterize SLE.

Could the numbers of CD4+ICOS^{high}CXCR5+ cells be increased simply as a result of T cell activation? Several findings are evidence against this explanation. First, CD84, CD200, CD57, and PD-1, all of which are more abundantly expressed by circulating Tfh cells than by other circulating CD4+ cells, are not known to be

markers of peripheral T cell activation. Thus, CD57+CXCR5+ cells as a proportion of CD4+ lymphocytes are increased in the circulating Tfh^{high} subset. CD57 is a Tfh cell–associated molecule, but it is not associated with cellular activation. Second, analysis of the lupus cohort by the use of CXCR5+ cells as a fraction of ICOS^{high}CD4+ cells identified the same circulating Tfh^{high} subset (median 24.3%, 15.8%, and 15.3%; $P = 0.0061$). Third, high levels of ICOS^{high}CD4+ cells were seen in an SLE patient in the group with high levels of circulating Tfh cells, revealing an imperfect correlation between ICOS expression and the size of the circulating Tfh cell fraction. Finally, an increased fraction of circulating Tfh cells segregated with particular clinical manifestations rather than with clinical and serologic markers of current disease activity.

In sanroque mice, Tfh cell dysregulation arises from a single-nucleotide change in the roquin gene (43). So far, we have not identified any mutations in the RC3H1 gene in SLE patients with high levels of circulating Tfh cells. Studies of sanroque mice revealed an important pathway for tolerance in GCs. We expect that defects in numerous genes could affect the function of this pathway to yield an autoimmune phenotype. Identification of a readily measurable biomarker of a breakdown in this pathway will enhance further studies aimed at identifying the underlying molecular or genetic defects.

Our view that circulating Tfh cells are related to GC Tfh cells is consistent with the observation that the numbers of circulating Tfh cells are also increased in a subset of patients with SS, a condition characterized by aberrant formation of GCs in nonlymphoid parenchyma (34,44). Salivary lymphocytes from patients with SS have been shown to have high levels of PD-1 (45). Given that ectopic GC expression occurs in SS, these data are consistent with an increased fraction of PD-1+ (ectopic) GC CD4+ T cells in a subset of patients. While some SLE and SS patients may have a shared pathway of GC T cell dysregulation, we favor the view that different manifestations of autoimmunity arise in the 2 conditions because of differences in other components of the pathway that leads to autoimmunity. For example, in SS, tertiary lymphoid tissue appears to be a key component of disease, but autoantibodies might differ because the antigen-presenting cells, such as salivary gland epithelial cells, are different from those that are typically encountered in secondary lymphoid tissue (34).

In conclusion, our results are consistent with the hypothesis that there is a distinctive GC pathway to disease in a subset of patients with SLE and that this pathway can be identified by the surrogate marker of high levels of circulating CD4+ T cells that resemble, and potentially originate from, Tfh cells. Further characterization of this cellular subset may provide an insight into the basic defect that causes autoimmunity in these patients and may provide an opportunity for more-targeted treatment of disease.

ACKNOWLEDGMENTS

We thank the SLE and SS patients, control subjects, and tonsillectomy patients who participated in our study, the staff of the Pathology Department at Canberra Hospital and Calvary Hospital for collecting the blood specimens, Dr. E. P. Chapman and colleagues at John James Hospital for their help with tonsil and blood specimen collection, Dr. T. Pham and colleagues at Canberra Hospital for help with tonsil and blood collection, and Drs. S. Riminton and S. Alexander (APOSLE investigators).

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Cook had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Simpson, Fulcher, Tangye, Goodnow, Vinuesa, Cook.

Acquisition of data. Simpson, Gatenby, Wilson, Malik, Fulcher, Manku, Vyse, Roncador, Huttley, Cook.

Analysis and interpretation of data. Simpson, Fulcher, Vyse, Goodnow, Vinuesa, Cook.

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