Human Blood CXCR5+CD4+ T Cells Are Counterparts of T Follicular Cells and Contain Specific Subsets that Differentially Support Antibody Secretion

Rimpei Morita,[1] Nathalie Schmitt,[1] Salah-Eddine Bentebibel,[1] Rajaram Ranganathan,[1] Laure Bourdery,[1] Gerard Zurawski,[1,2] Emile Foucat,[1] Melissa Dullaers,[1] SangKon Oh,[1,2] Natalie Sabzghabaei,[1] Elizabeth M. Lavecchio,[1] Marilynn Punaro,[3,4] Virginia Pascual,[1,3] Jacques Banchereau,[1,2,5,*] and Hideki Ueno[1,2,*]

[1]Baylor Institute for Immunology Research, Baylor Research Institute, Dallas, TX 75204, USA
[2]INSERM U899, Dallas, TX 75204, USA
[3]Texas Scottish Rite Hospital for Children, Dallas, TX 75219, USA
[4]UT Southwestern Medical Center and Children's Medical Center, Dallas, TX 75235, USA
[5]Department of Gene and Cell Medicine and Department of Medicine, Immunology Institute, Mount Sinai School of Medicine, New York, NY 10029, USA
*Correspondence: jacquesb@baylorhealth.edu (J.B.), hidekiu@baylorhealth.edu (H.U.)
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SUMMARY

Although a fraction of human blood memory CD4+ T cells expresses chemokine (C-X-C motif) receptor 5 (CXCR5), their relationship to T follicular helper (Tfh) cells is not well established. Here we show that human blood CXCR5+CD4+ T cells share functional properties with Tfh cells and appear to represent their circulating memory compartment. Blood CXCR5+CD4+ T cells comprised three subsets: T helper 1 (Th1), Th2, and Th17 cells. Th2 and Th17 cells within CXCR5+, but not within CXCR5−, compartment efficiently induced naive B cells to produce immunoglobulins via interleukin-21 (IL-21). In contrast, Th1 cells from both CXCR5+ and CXCR5− compartments lacked the capacity to help B cells. Patients with juvenile dermatomyositis, a systemic autoimmune disease, displayed a profound skewing of blood CXCR5+ Th cell subsets toward Th2 and Th17 cells. Importantly, the skewing of subsets correlated with disease activity and frequency of blood plasmablasts. Collectively, our study suggests that an altered balance of Tfh cell subsets contributes to human autoimmunity.

INTRODUCTION

Antibody responses are largely dependent on the help provided by CD4+ T cells. CD4+ T cells are fundamental for the generation of germinal centers (GCs), a discrete structure in secondary lymphoid organs where selection of high-affinity B cells and development of B cell memory occur (Allen et al., 2007; MacLennan, 1994). Recently, CD4+ T cells present in B cell follicles, named T follicular helper (Tfh) cells, have been established as a T helper (Th) cell subset specialized for providing help to B cells in GCs (Fazilleau et al., 2009; King et al., 2008). Tfh cells express the chemokine (C-X-C motif) receptor 5 (CXCR5)

(Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000), which allows their migration into B cell follicles in response to the specific ligand CXCL13. Tfh cells secrete IL-4, IL-10, and IL-21, cytokines that promote growth, differentiation, and class switching of B cells (Ettinger et al., 2005; Good et al., 2006; Pene et al., 2004). Tfh cells also express surface molecules essential for helper functions, including CD40-ligand (CD40L) and inducible costimulator (ICOS) (King et al., 2008). Tfh cells express large amounts of B cell lymphoma 6 (Bcl-6) (Chtanova et al., 2004; Rasheed et al., 2006), which is necessary and sufficient for the development of Tfh cells in vivo (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). In contrast, B lymphocyte-induced maturation protein 1 (Blimp-1), a transcription repressor that regulates the function of Bcl-6, inhibits the generation of Tfh cells (Johnston et al., 2009). Thus, Tfh cell generation is controlled by the balance of these two transcription repressors. This supports the hypothesis that the developmental pathway of Tfh cells is distinct from that of other canonical Th cell subsets (Nurieva et al., 2008). Alternatively, there is evidence that mouse Tfh cells are heterogeneous and encompass distinct subsets secreting cytokines characteristic of Th1, Th2, and Th17 cells (Bauquet et al., 2009; Fazilleau et al., 2009; King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). Furthermore, mouse Th2 (Zaretsky et al., 2009) and Treg (Tsuji et al., 2009) cells were shown to be convertible into Tfh cells in vivo. Therefore, the relationship between Tfh cells and other Th cell subsets still remains unclear. Notably, whereas all these studies were performed with inbred mouse strains, whether Tfh cells in humans are comprised of different subsets is largely unknown. Previous studies have shown that tonsillar Tfh cells display distinct phenotype and genetic profiles from other canonical Th cell subsets (Chtanova et al., 2004; Kim et al., 2004; Rasheed et al., 2006). However, as suggested in mouse studies, the precursors of Tfh cells might be composed of heterogeneous cell populations also in humans, and they might differentiate into distinct types of Tfh cells.

Furthermore, although several mouse studies show that over-representation of Tfh cells is associated with the development of systemic autoimmunity (Linterman et al., 2009; Subramanian et al., 2006; Vinuesa et al., 2005), their association with human

autoimmune diseases remains largely unknown. Patients with autoimmune diseases such as lupus or rheumatoid arthritis display high-affinity somatically mutated autoantibodies in sera (Mietzner et al., 2008; Shlomchik et al., 1987), suggesting the involvement of Tfh cells (or Tfh-committed extrafollicular cells [Poholek et al., 2010]) in the pathogenesis. Although a systematic approach would be required to define the role of Tfh cells in human autoimmune diseases, obtaining lymph node samples from patients routinely and/or longitudinally is extremely challenging. Therefore, there is a strong need to establish surrogate strategies to assess the quality of Tfh cell responses in humans. In this regard, analysis of blood CD4$^+$ T cells expressing CXCR5 (Forster et al., 1994) might facilitate such studies. Several observations suggest a relationship between CXCR5$^+$CD4$^+$ T cells and Tfh cells. For example, humans who show severely impaired GC formation through deficiency of CD40-ligand or ICOS display substantially fewer circulating CXCR5$^+$CD4$^+$ T cells (Bossaller et al., 2006). On the contrary, CXCR5$^+$CD4$^+$ T cells expressing ICOS are present at a higher frequency in blood of lupus patients (Simpson et al., 2010). However, whether circulating CXCR5$^+$CD4$^+$ T cells indeed share the phenotypic and functional properties of Tfh cells remains to be established.

Here we show that human blood CXCR5$^+$CD4$^+$ T cells appear to represent a circulating pool of memory Tfh cells and can be distinguished into Th1, Th2, and Th17 cell subsets with different capacities to regulate B cell responses. We further show an alteration of blood CXCR5$^+$CD4$^+$ T cell subsets in an autoimmune disease, juvenile dermatomyositis (JDM).

RESULTS

Human Blood CXCR5$^+$CD4$^+$ T Cells Induce the Differentiation of Naive B Cells toward Plasmablasts

In healthy adult blood, CXCR5 was expressed by 8.3% ± 1.8% of CD4$^+$ T cells and 18.9% ± 3.6% of memory (CD45RA$^-$) CD4$^+$ T cells (mean ± SD, n = 10) (Figure 1A). Consistent with previous observations (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000), blood CXCR5$^+$CD4$^+$ T cells expressed CCR7 and CD62L, but few expressed activation molecules expressed by Tfh cells, such as ICOS and CD69 (Figure S1A available online), suggesting a resting state. Both CXCR5 and CCR7 were functional, as shown by the fact that blood CXCR5$^+$CD4$^+$ T cells migrated in response to the ligands CXCL13 and CCL19, respectively (Figure S1B).

To determine their capacity to help B cells, memory (CD45RA$^-$) CXCR5$^+$ cells were sorted and cultured with autologous naive B (IgD$^+$CD27$^-$CD19$^+$) cells. Naive (CD45RA$^+$) and memory CXCR5$^-$CD4$^+$ T cells were also sorted for comparison. To mimic the antigen-specific interaction between T and B cells, staphylococcal enterotoxin B (SEB), a superantigen, was added to the cultures. Naive CD4$^+$ T cells did not induce naive B cells to produce immunoglobulins (Igs) (Figure 1B). Memory CXCR5$^-$CD4$^+$ T cells induced naive B cells to produce only low amounts of IgM, but no IgG or IgA. In contrast, CXCR5$^+$CD4$^+$ T cells were potent at inducing naive B cells to produce IgM, IgG, and IgA (Figure 1B). The Ig production was totally dependent on cognate interactions between T and B cells, as shown by the fact that naive B cells cocultured with CXCR5$^+$CD4$^+$ T cells did not produce Igs in the absence of SEB (Figure 1C). CXCR5$^+$CD4$^+$

T cells were also more efficient than CXCR5$^-$CD4$^+$ T cells in inducing memory B cells to produce Igs (Figure 1D).

Kinetics studies revealed that CXCR5$^+$CD4$^+$ T cells induced naive B cells to produce IgG and IgA as early as day 6 of culture, whereas CXCR5$^-$CD4$^+$ T cells did not induce either IgG or IgA secretion even at day 12 (Figure 1E). The number of viable T cells was similar between the cultures of CXCR5$^-$ and CXCR5$^+$ CD4$^+$ T cells with naive B cells (Figures 1F, top, and Figure 1G), indicating that the inability of CXCR5$^-$CD4$^+$ T cells to induce naive B cells to produce IgG or IgA was not due to their poor survival. In contrast, the number of viable B cells was constantly higher when naive B cells were cultured with CXCR5$^+$ cells than with CXCR5$^-$CD4$^+$ T cells (Figure 1F, bottom). Of note, the number of viable B cells decreased after day 5 even when cultured with CXCR5$^+$CD4$^+$ T cells, suggesting that the T cells could not fully support the survival of B cells in vitro. Nonetheless, culturing naive B cells with CXCR5$^+$CD4$^+$ T cells yielded higher numbers of CD38$^+$CD19^{lo} cells than with CXCR5$^-$CD4$^+$ T cells (Figures 1H and 1I), indicating that a fraction of surviving B cells differentiated into plasmablasts. Consistently, CXCR5$^+$ CD4$^+$ T cells efficiently induced naive B cells to express activation-induced cytidine deaminase (AID, encoded by *AICDA* gene), a factor required for class switching, and Blimp-1 (encoded by *PRDM1* gene), a transcription factor critical for the differentiation of plasma cells, but not Bcl6 (encoded by *BCL6* gene) (Figure 1J).

Collectively, these observations show that blood CXCR5$^+$ CD4$^+$ T cells were more efficient than CXCR5$^-$CD4$^+$ T cells at inducing naive B cells to differentiate into plasmablasts and to promote class switching.

Blood CXCR5$^+$CD4$^+$ T Cells Help Naive B Cells through IL-21

Similar to tonsillar Tfh cells, blood CXCR5$^+$CD4$^+$ T cells secreted IL-21 upon contact with naive B cells, whereas CXCR5$^-$CD4$^+$ T cells barely secreted IL-21 (Figure 2A). Although CXCR5$^+$CD4$^+$ T cells secreted IL-21 within 24 hr after interaction with naive B cells, very low amounts, if any, of IL-21 was secreted by CXCR5$^-$CD4$^+$ T cells up to 96 hr (Figure S2A). Notably, CXCR5$^+$ CD4$^+$ T cells also secreted larger amounts of CXCL13 than CXCR5$^-$CD4$^+$ T cells (Figure 2B), a chemokine produced by tonsillar Tfh cells (Kim et al., 2004; Rasheed et al., 2006).

Previous studies demonstrated that IL-21 secreted by tonsillar Tfh cells plays a central role in the expansion and plasma cell differentiation of cocultured B cells (Bryant et al., 2007). Similarly, blocking IL-21 during the coculture of naive B cells with blood CXCR5$^+$CD4$^+$ T cells resulted in a dose-dependent inhibition of Ig secretion (Figure 2C) and B cell recovery (Figure 2D). Conversely, addition of IL-21 resulted in the enhancement of IgM secretion as well as the induction of IgG and IgA secretion by naive B cells cultured with CXCR5$^-$, but not naive, CD4$^+$ T cells (Figure 2E). Consistent with previous studies with Tfh cells (Bauquet et al., 2009; Odegard et al., 2008; Vogelzang et al., 2008), B cell help by blood CXCR5$^+$CD4$^+$ T cells was also dependent on ICOS, as shown by the fact that blocking of ICOS-ICOS ligand interaction inhibited both IL-21 secretion (Figure S2B) and Ig secretion (Figure 2F). Large amounts of IL-10 was also detected in the cocultures of CXCR5$^+$CD4$^+$ T cells and naive
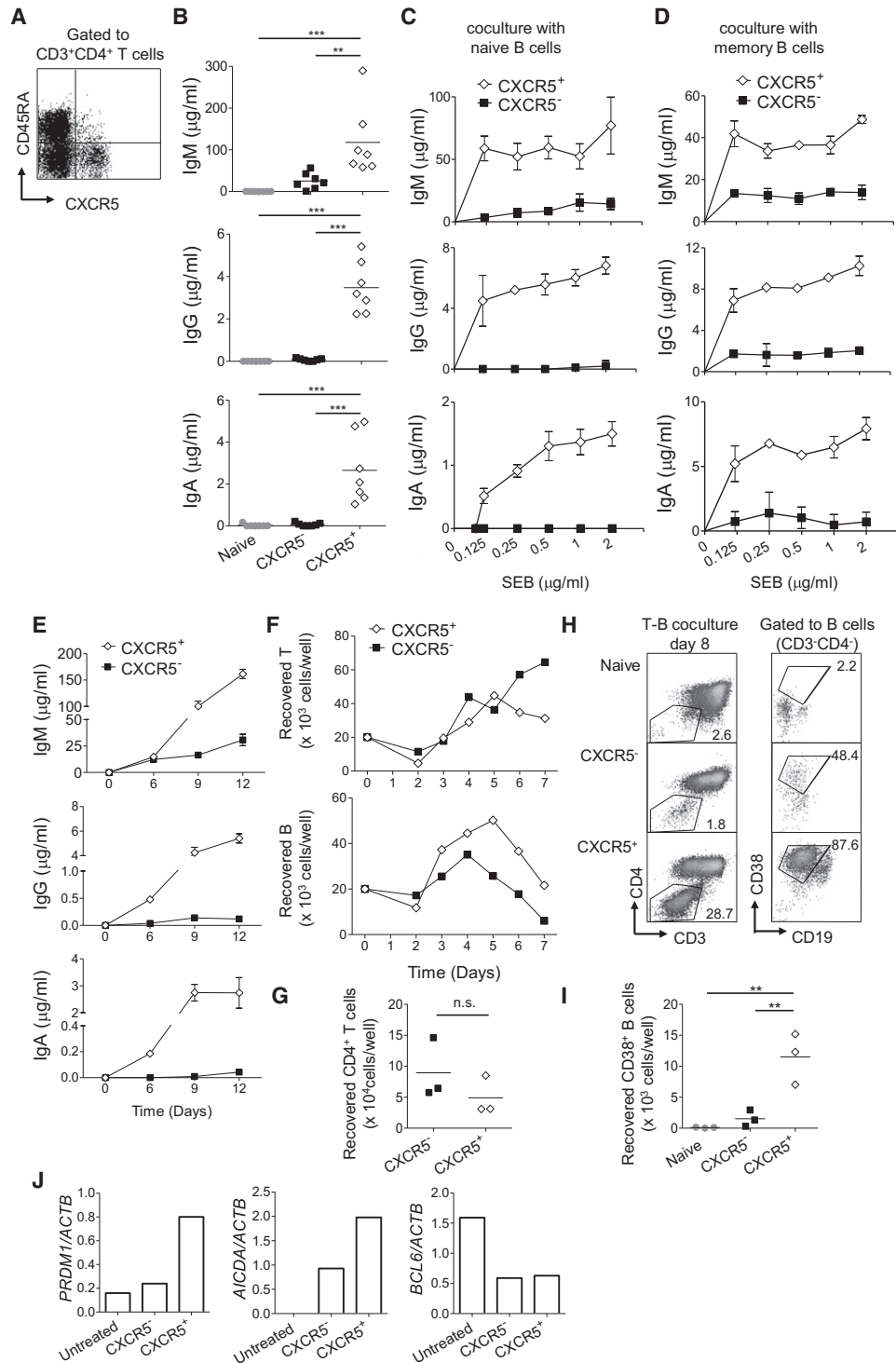
Figure 1. Blood CXCR5⁺CD4⁺ T Cells Induce Naive B Cells to Differentiate into Ig-Producing Plasmablasts

(A) CXCR5 expression by blood CD4⁺ T cells. PBMCs were stained with CD3, CD4, CD45RA, and CXCR5 mAbs. Gated to CD3⁺CD4⁺ T cells.

(B) Blood naive, CXCR5⁻, and CXCR5⁺CD4⁺ T cells were cultured with autologous naive B cells in the presence of SEB. Ig concentrations were measured at day 12. One-way ANOVA test. Data from seven independent experiments. **p < 0.01, ***p < 0.001.

(C and D) CXCR5⁻ or CXCR5⁺CD4⁺ T cells were cultured with naive (C) or memory (D) B cells in the presence of titrated doses of SEB. Ig concentrations at day 12 in (C) and day 6 in (D). n = 3, mean ± SD. Representative data from three independent experiments.

(E) Ig concentrations at different time points in the cultures of CXCR5⁻ or CXCR5⁺CD4⁺ T cells with naive B cells. n = 3, mean ± SD. Representative data from four independent experiments.

(F) Number of viable CD4⁺ T and B cells at different time points. Representative data from two independent experiments.

B cells (Figure 2G), and blocking IL-10 resulted in a partial inhibition of Ig secretion (Figure 2H).

Whether blood CXCR5$^+$CD4$^+$ T cells contain antigen-specific memory cells has been controversial (Breitfeld et al., 2000; Rivino et al., 2004; Schaerli et al., 2001). To address this issue, isolated CXCR5$^-$ and CXCR5$^+$CD4$^+$ T cells were cocultured with autologous monocytes that had been pulsed with inactivated influenza (flu) virus, or cytomegalovirus, and the proliferation of T cells was analyzed at day 5. Whereas CXCR5$^-$CD4$^+$ T cells proliferated robustly in response to the stimulation with both viruses, these stimulations also induced CXCR5$^+$CD4$^+$ T cells to proliferate (Figure 2I) and to secrete cytokines, including IL-2 and IFN-γ (Figure 2J). Notably, CXCR5$^+$CD4$^+$ T cells secreted more IL-2 than CXCR5$^-$CD4$^+$ T cells in response to the stimulation with virus antigens (Figure 2J). To directly illustrate the flu-specific CXCR5$^+$CD4$^+$ T cells, PBMCs obtained from healthy donors (who did not receive influenza vaccines more than 1 year) were incubated for 6 hr with either a seasonal flu vaccine (Fluzone) or a heat-inactivated flu virus (PR8). Then cells were analyzed for the expression of CD4 and CXCR5 together with the expression of intracytoplasmic CD154, which permits the sensitive identification of antigen-specific CD4$^+$ T cells (Chattopadhyay et al., 2006). Whereas CXCR5 is expressed by activated CD4$^+$ T cells (Schaerli et al., 2001), CXCR5$^-$CD4$^+$ T cells stimulated for 6 hr with SEB remained negative for CXCR5 expression (Figure S2C). As shown in Figure 2K, flu-specific CD4$^+$ T cells were detected as CD154$^+$ cells in both stimulations, which contained CXCR5$^+$ cells. Furthermore, a fraction of flu antigen-specific (CD154$^+$) CXCR5$^+$CD4$^+$ T cells also expressed intracytoplasmic IL-2 and/or IFN-γ upon stimulation (Figure S2D).

Collectively, these observations show that CXCR5$^+$CD4$^+$ T cells shared functional properties of Tfh cells. Inasmuch as CXCR5$^+$CD4$^+$ T cells contained antigen-specific memory cells, blood CXCR5$^+$CD4$^+$ T cells appear to represent circulating memory Tfh cells.

Three Distinct Th Cell Subsets in Human Blood

The expression of chemokine receptors has been instrumental for defining human CD4$^+$ T cell subsets. The expression of CXCR3 is preferentially maintained by cells committed to the Th1 cell pathway (Bonecchi et al., 1998; Rabin et al., 2003; Sallusto et al., 1998), whereas CCR6 is expressed by Th17 cells (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Singh et al., 2008). Though blood CXCR5$^+$CD4$^+$ T cells were previously shown to coexpress other chemokine receptors (Lim et al., 2008), the relationship between chemokine receptor expression and their function has not been established. As illustrated in Figure 3A, differential expression of CXCR3 and CCR6 defined three major subsets within blood CXCR5$^+$CD4$^+$ T cells: CXCR3$^+$CCR6$^-$, CXCR3$^-$CCR6$^-$, and CXCR3$^-$CCR6$^+$ cells. CXCR5$^-$CD4$^+$ T cells contained four subpopulations including CXCR3$^+$

CCR6$^+$ cells. When compared to CXCR5$^+$CD4$^+$ T cells, the pool of CXCR5$^-$CD4$^+$ T cells contained fewer CXCR3$^-$CCR6$^+$ T cells (p < 0.01, paired t test) and more CXCR3$^+$CCR6$^+$ T cells (p < 0.001) (Figure 3B).

To analyze the functional differences of blood memory CD4$^+$ T cell populations, seven major subpopulations (four CXCR5$^-$ and three CXCR5$^+$) were isolated according to the expression of CXCR5, CXCR3, and CCR6. To analyze cytokine secretion patterns, each subpopulation was cocultured for 2 days with SEB-pulsed naive B cells. As expected (Figure 2A), all four subpopulations within the CXCR5$^-$ compartment secreted very little, if any, IL-21 upon interaction with naive B cells (Figure 3C). Within the CXCR5$^+$CD4$^+$ T cell compartment, only CXCR3$^-$CCR6$^-$ and CXCR3$^-$CCR6$^+$ cells produced IL-21. Measurement of other cytokines revealed that each cell population secreted different sets of cytokines (Figure 3D). CXCR3$^+$CCR6$^-$ cells in both CXCR5$^+$ and in CXCR5$^-$ compartments secreted IFN-γ but not Th2 or Th17 cytokines. Th2 cytokines, i.e., IL-4, IL-5, and IL-13, were exclusively secreted by CXCR3$^-$CCR6$^-$ cells, whereas Th17 cytokines IL-17A and IL-22 were produced by CXCR3$^-$CCR6$^+$ cells, in both CXCR5$^+$ and CXCR5$^-$ cells. Expression profiling of transcription factors demonstrated that within the CXCR5$^+$CD4$^+$ T cell compartment, CXCR3$^+$CCR6$^-$ cells expressed T-bet, a transcription factor of Th1 cells, CXCR3$^-$CCR6$^-$ cells expressed GATA3, a transcription factor of Th2 cells, whereas CXCR3$^-$CCR6$^+$ cells expressed RORγT, a transcription factor of Th17 cells (Figure 3E). The similar pattern of transcription factor expression was also observed within CXCR5$^-$CD4$^+$ T cell compartment. Thus, both blood CXCR5$^+$ and CXCR5$^-$CD4$^+$ T cells included Th1, Th2, and Th17 cells.

Next we analyzed the expression of *BCL6* and *PRDM1* transcripts in blood memory Th cell subsets with real-time RT-PCR. Several reports demonstrated that blood CXCR5$^+$CD4$^+$ T cells express much lower amounts of *BCL6* transcript than tonsillar Tfh cells (Chtanova et al., 2004; Rasheed et al., 2006; Simpson et al., 2010). Consistently, the expression of *BCL6* transcript was similar among CXCR5$^+$ and CXCR5$^-$ Th cell subsets (Figure 3F). However, *PRDM1* transcript expression was lower in all the CXCR5$^+$ Th cell subsets than in their counterparts in CXCR5$^-$ cells. Thus, the balance between Bcl-6 and Blimp-1 appears to be differentially regulated between CXCR5$^+$ and CXCR5$^-$ Th cell subsets.

CXCR5$^+$ Th2 and CXCR5$^+$ Th17 Cells Help B Cell Differentiation

We next examined the ability of the seven blood CD4$^+$ T cell populations to help naive B cells. The sorted CD4$^+$ T cells were cultured with SEB-pulsed naive B cells for 12 days, and secreted Ig concentrations were measured. Both CXCR5$^+$ and CXCR5$^-$ Th1 (CXCR3$^+$CCR6$^-$) cells failed to induce naive B cells to produce Igs (Figures 4A and 4B). CXCR5$^+$ Th1 cells were also incapable of inducing memory B cells to produce Igs

(G) Number of viable CD4$^+$ T cells at day 8. Paired t test. Data from three independent experiments.
(H) CD38$^+$ plasmablast population in the coculture of blood Th cell subsets and naive B cells at day 8. Representative data from three independent experiments.
(I) Number of plasmablasts in the cocultures. One-way ANOVA test. Data from three independent experiments.
(J) Measurement of *BCL6*, *PRDM1*, and *AICDA* mRNA expression by real-time RT-PCR in naive B cells cultured with blood Th cell subsets. Expression of each mRNA was normalized to that of *ACTB* mRNA. Before and after 7 day culture with CXCR5$^-$ or CXCR5$^+$CD4$^+$ T cell subsets (B cells were purified after culture). Representative data from two independent experiments.
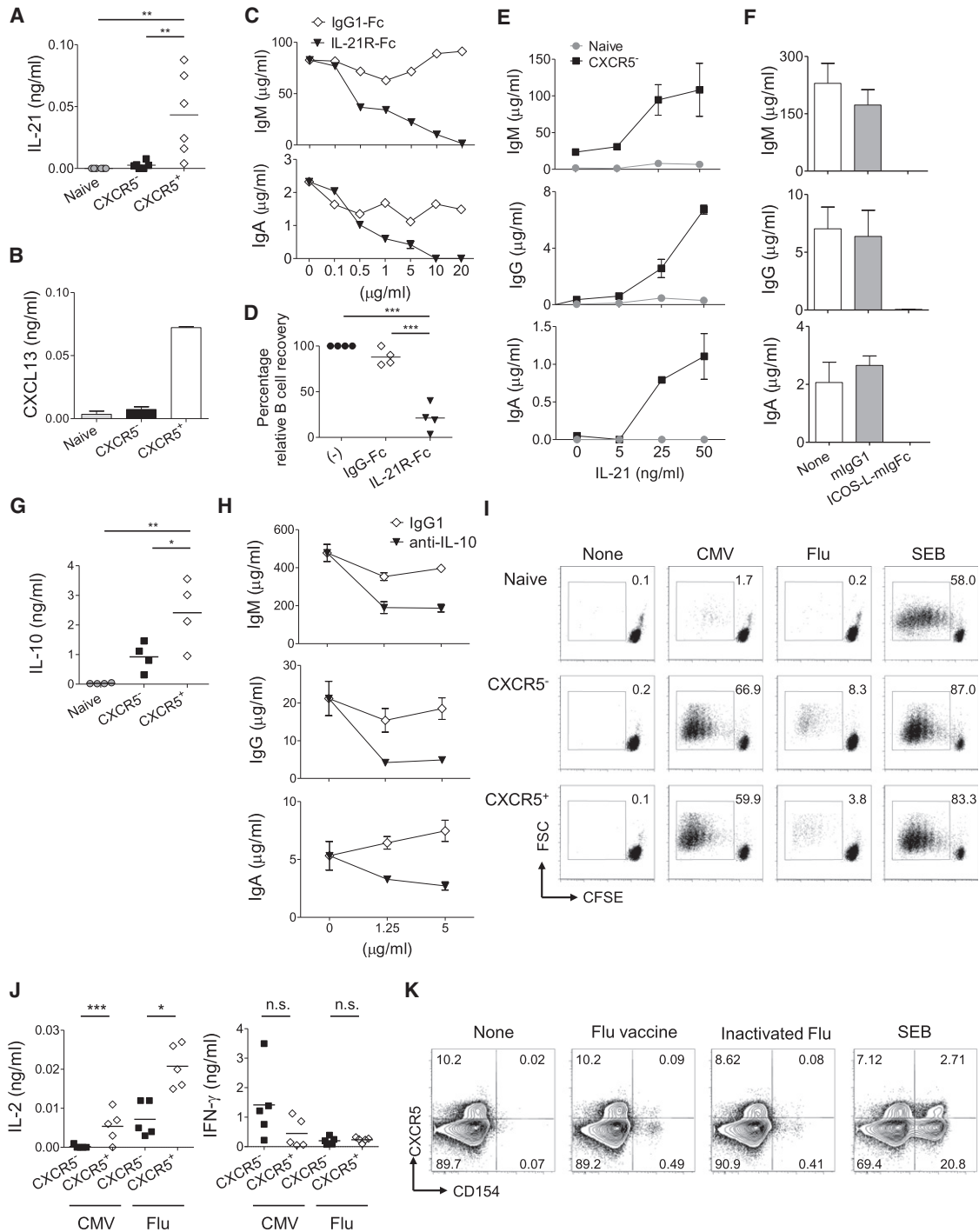
Figure 2. Blood CXCR5+CD4+ T Cells Depends on IL-21, IL-10, and ICOS for B Cell Help

(A) IL-21 secretion by blood Th cell subsets cultured with naive B cells. Data from six independent experiments. One-way ANOVA test. **$p < 0.01$.

(B) CXCL13 secretion by blood Th cell subsets cultured with naive B cells. n = 3. Mean ± SD. Representative data from three independent experiments.

(C) Titrated amounts of IL-21R-Fc were added to the coculture of CXCR5+CD4+ T cells and naive B cells. Ig concentrations at day 12. IgG is not shown because of the cross-reactivity to the Fc portion of IL-21R-Fc. Representative data from two independent experiments.

(D) Recovery of viable B cells at day 12. Normalized to the culture of CXCR5+CD4+ T cells and naive B cells. One-way ANOVA test, n = 4. ***$p < 0.001$.

(E) Titrated amounts of IL-21 were added to the coculture of naive or CXCR5−CD4+ T cells with naive B cells. Ig concentrations at day 12. n = 3, mean ± SD. Representative data from three independent experiments.

(F) Ig concentrations at day 12 in the coculture of CXCR5+CD4+ T cells and naive B cells with an ICOS-blocking reagent. n = 3; mean ± SD. Representative data from two independent experiments.

(Figure S3A). Among Th2 (CXCR3⁻CCR6⁻) cells, CXCR5⁺ cells induced naive B cells to produce IgM, IgG, IgA, and IgE. In contrast, CXCR5⁻ Th2 cells induced B cells to secrete only IgM and small amounts of IgE, but virtually no IgG and IgA (Figures 4A and 4B). Among Th17 (CXCR3⁻CCR6⁺) cells, CXCR5⁺ cells potently induced naive B cells to produce IgM, IgG, and IgA, but not IgE. However, CXCR5⁻ Th17 cells completely lacked the capacity to help naive B cells (Figures 4A and 4B). The inability of CXCR5⁺ Th1 cells to help naive B cells was not due to the poor survival of T cells in the cultures (Figure 4C), but due to the lack of capacity to maintain the survival of B cells (Figure 4D). In contrast, CXCR5⁺ Th2 and Th17 cells efficiently induced naive B cells to proliferate and to differentiate into CD19^lo^CD38⁺ plasmablasts (Figure 4D).

Whereas CXCR5⁺ Th2 cells and Th17 cells induced naive B cells to produce comparable amounts of IgG (Figure 4B) and its subclasses (Figure S3B), CXCR5⁺ Th17 cells induced naive B cells to produce higher amounts of IgA (Figure 4B), in particular IgA2 (Figure 4E), than did CXCR5⁺ Th2 cells. Blocking IL-21 in the culture of naive B cells with CXCR5⁺ Th2 cells resulted in a substantial decrease in IgM and IgG3 (the IgG isotype that does not crossreact to IL-21R-Fc) production (Figure 4F), whereas blocking IL-4 resulted in a substantial inhibition of IgE production (Figure 4G). The help of CXCR5⁺ Th17 cells to naive B cells was largely dependent on IL-21, as shown by the fact that blocking IL-21 strongly inhibited the production of IgM, IgG3, and IgA (Figure 4F).

Thus, the capacity to induce naive B cells to differentiate into Ig-producing cells was different among blood CD4⁺ T cell populations.

CXCR5⁺ Th Cell Subsets Are Altered in Juvenile Dermatomyositis

The identification of functionally distinct Th cell subsets within blood CXCR5⁺ compartment led us consider that their analysis might reveal dysregulation of Tfh cell responses in autoimmune diseases. Juvenile dermatomyositis (JDM) is a chronic, multi-system autoimmune disease involving muscle, skin, gastrointestinal tract, and other organs. JDM patients with active disease typically show proximal muscle weakness and skin rash (Feldman et al., 2008; Suber et al., 2008). Studies on JDM have revealed several mediators common to systemic lupus erythematosus, including type I IFN (Walsh et al., 2007). Autoantibodies can be found in JDM patients' serum, though relatively little is known regarding their specificities (Suber et al., 2008). The pathogenesis of JDM remains largely unknown.

We analyzed the blood CXCR5⁺ T cell subsets in samples from JDM patients (total 52 samples from 45 patients) and age-matched healthy pediatric controls (43 donors) (summa-rized in Table 1). Blood samples were also obtained from age-matched pediatric patients with psoriatic arthritis (PSOA, 31 patients), a systemic inflammatory disease mediated by inflammatory T cells (Lewkowicz and Gottlieb, 2004). Thirty-five JDM patients were under standard treatment including corticosteroids, methotrexate, Etanercept (TNF antagonist), and/or high-dose immunoglobulin (Table S1). Twenty-six samples were obtained from symptomatic JDM patients who displayed skin rash and/or muscular weakness (measured by the childhood myositis assessment scale [CMAS]) at the time of sampling. These patients included four untreated active patients (Table S1).

The frequency of CXCR5⁺ cells within CD4⁺ T cells was not substantially different among the three groups (Figure 5A). However, the frequency of Th1 cells within the CXCR5⁺CD4⁺ T cell compartment was significantly lower in JDM patients when compared to PSOA patients and healthy controls (Figure 5B; JDM 23.5% ± 0.8%, PSOA 32.8% ± 1.3%, and control 32.4% ± 1.0%; mean ± SEM; both p < 0.0001, one-way ANOVA test). In contrast, the frequencies of Th2 and Th17 cells within CXCR5⁺CD4⁺ T cells were significantly higher in JDM patients compared to PSOA patients and healthy controls (Th2: JDM 29.4% ± 1.0%, PSOA 24.4% ± 1.0%, and control 23.7% ± 0.8%; both p < 0.0001; Th17: JDM 35.8% ± 1.0%, PSOA 27.9% ± 1.0%, and control 28.1% ± 1.0%; both p < 0.0001). The skewing of CXCR5⁺ Th cell subsets resulted in a significant increase in B helpers over non-B helpers in JDM, as determined by the ratio of Th2+Th17 (B helpers) over Th1 (non B helpers) cells (Figure 5C; JDM 3.1 ± 0.2, PSOA 1.7 ± 0.1, and control 1.7 ± 0.1; mean ± SEM; both p < 0.0001). The Th cell subsets within the CXCR5⁻ compartment were also skewed toward Th2 and Th17 cells in JDM patients (Figure S4A). Of note, in the PSOA group, patients receiving methotrexate or Etanercept showed comparable frequencies of the CXCR5⁺ Th cell subsets, indicating that these treatments did not alter the composition of CXCR5⁺ Th cell subsets (Figure S4B).

Thus, blood CXCR5⁺ Th cell subsets were skewed toward Th2 and Th17 cells in JDM patients.

Tfh Cell Subset Skewing Is Associated with Disease Activity

To determine whether the skewing in Th cell subsets is associated with disease activity in JDM, patients were subgrouped according to the severity of clinical manifestations. Active patients with skin rash and muscular weakness showed a lower frequency of Th1 cells within CXCR5⁺ cells than did asymptomatic patients (Figure 5D). Accordingly, patients with skin rash and muscular weakness displayed a higher ratio of Th2+Th17/Th1 cells in CXCR5⁺ cells (Figure 5D). The skewing

(G) IL-10 secretion in supernatants of blood Th cell subsets cultured with naive B cells. Data from four independent experiments. One-way ANOVA test. *p < 0.05, **p < 0.01.

(H) Ig concentrations at day 12 in the coculture of CXCR5⁺CD4⁺ T cells and naive B cells with indicated amounts of anti-IL-10. n = 3, mean ± SD. Representative data from three independent experiments.

(I) CFSE-labeled blood Th cell subsets were cultured with autologous monocytes incubated with inactivated flu virus or CMV. Cell proliferation was analyzed at day 5. Representative data from two independent experiments.

(J) IL-2 and IFN-γ secretion in supernatants at day 2. Data from five independent experiments. Paired t test. ***p < 0.001, *p < 0.05.

(K) PBMCs were stimulated with none, flu vaccine, inactivated flu virus, or SEB for 6 hr in the presence of Brefeldin A and monensin, and the intracytoplasmic expression of CD154 in CXCR5⁺ or CXCR5⁻CD4⁺ T cells was analyzed. Representative data from four independent experiments.
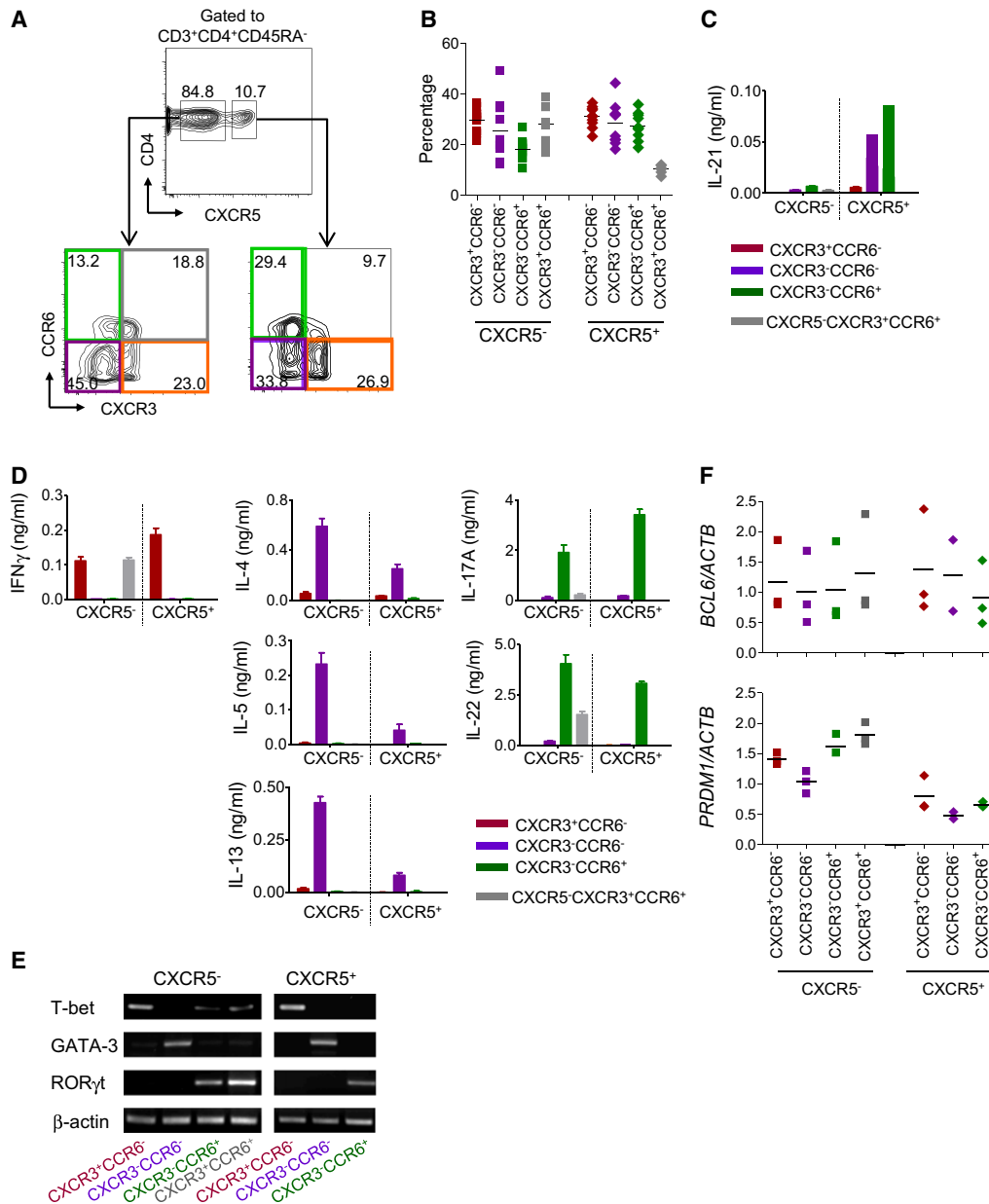
Figure 3. Blood CXCR5⁺CD4⁺ T Cells Are Composed of Th1, Th2, and Th17 Cells

(A) CXCR3 and CCR6 expression on blood CXCR5⁻ or CXCR5⁺CD4⁺ T cell population. Gated to CD3⁺CD4⁺CD45RA⁻ cells.

(B) Frequency of populations within blood CXCR5⁻ and CXCR5⁺CD4⁺ T cells of ten healthy adults.

(C) The seven blood Th cell populations were cocultured with naive B cells and the secreted IL-21 was measured at 48 hr. n = 3, mean ± SD. Representative data from four independent experiments.

(D) Other cytokine secretion in the cocultures of the seven blood Th cell populations and naive B cells. n = 3, mean ± SD. Representative data from four independent experiments.

(E) Expression of each transcriptional factor in the seven blood Th cell populations was assessed by RT-PCR. Representative data from two independent experiments.

(F) The seven Th cell populations were sorted from PBMCs of three donors, and expression of *BCL6* and *PRDM1* mRNA was analyzed by real-time RT-PCR. Normalized to *ACTB* mRNA expression in each Th cell subset.

of Th cell subsets is not due to the treatment, as shown by the fact that neither the frequency of Th1 cells nor the ratio of Th2+Th17/Th1 cells in CXCR5⁺ cells were different among active patients receiving intravenous corticosteroids, high-dose intravenous immunoglobulins, or no treatment (Figure 5E). Further-

more, patients with skin rash and muscular weakness displayed a significant increase in the absolute number of CXCR5⁺ Th2 and Th17 cells in blood, when compared to healthy controls (Figure 5F; Th2: active JDM [n = 25] 2.0 ± 0.2 versus healthy [n = 17] 1.3 ± 0.1 × 10⁶ cells/L; mean ± SEM; p = 0.001,
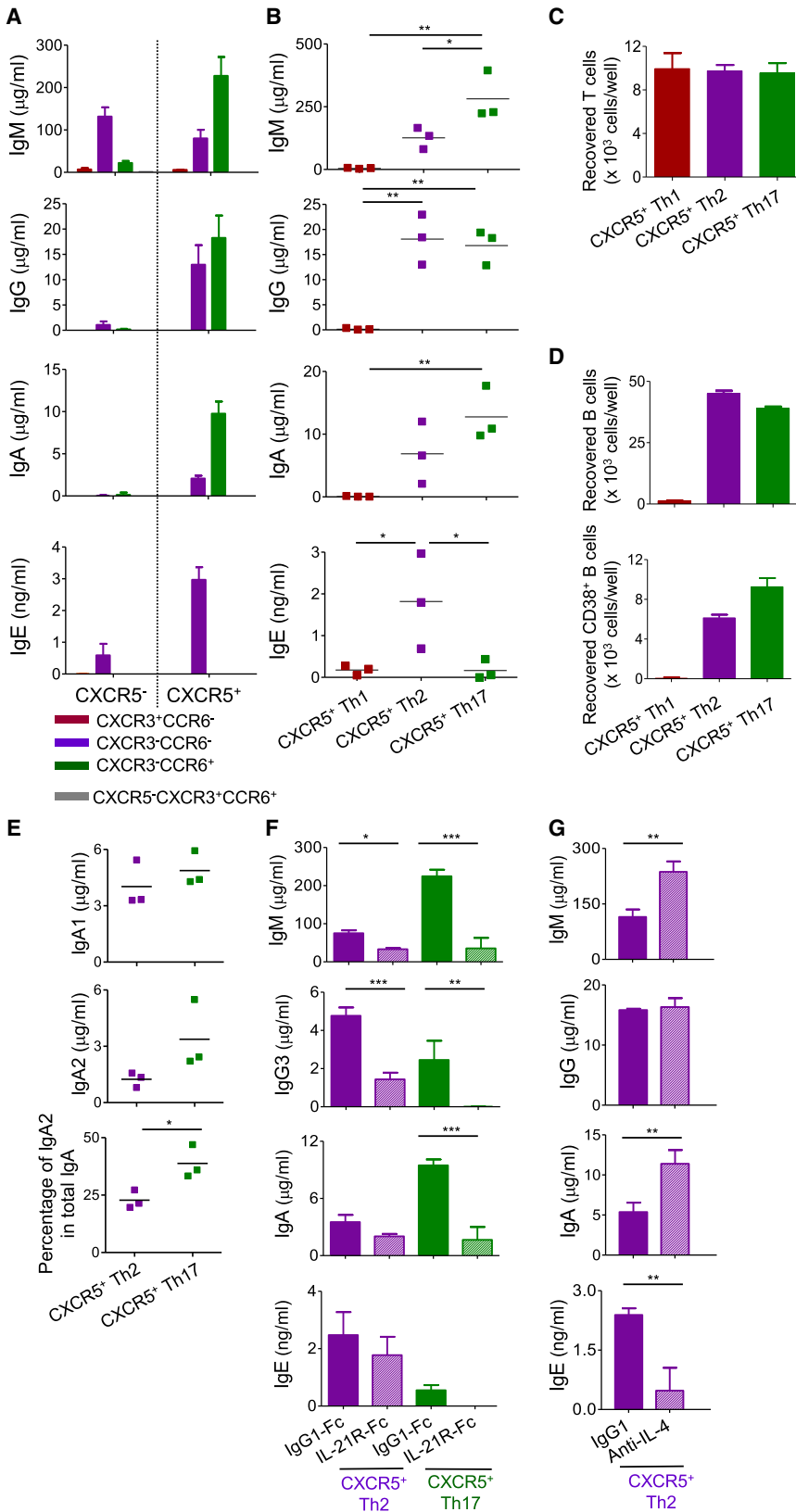
Figure 4. CXCR5+ Th2 and Th17 Cells Efficiently Help Naive B Cells

(A) Ig secretion by naive B cells cocultured with the seven blood Th cell populations for 12 days. n = 3–4, mean ± SD. Representative data from three independent experiments.

(B) Ig secretion from naive B cells cocultured with blood CXCR5+ Th cell subsets. Data of three independent experiments. One-way ANOVA test. *p < 0.05, **p < 0.01.

(C) Number of viable CD4+ T cells at day 8. n = 3–4, mean ± SD. Representative data from two independent experiments.

(D) Number of viable B cells and plasmablasts at day 8. n = 3–4, mean ± SD. Representative data from two independent experiments.

(E) IgA1 and IgA2 production. Data from three independent experiments. Student's t test. *p < 0.05.

(F) Addition of IL-21R-Fc chimera protein to the cocultures of naive B cells and CXCR5+ Th2 or Th17 cells. n = 3, mean ± SD. Student's t test. *p < 0.05, **p < 0.01, ***p < 0.001. Representative data from three independent experiments.

(G) Addition of IL-4 blocking antibody to the culture of CXCR5+ Th2 cells and naive B cells. n = 3, mean ± SD. Student's t test. **p < 0.01. Representative data from two independent experiments.

Table 1. Summary of Patients and Donors in the Study

	JDM (52 Samples)				PSOA (31 Samples)		Health (43 Samples)		p Value (t Test)				
	Symptomatic		Asymptomatic						JDM Symptomatic versus Asymptomatic	JDM Symptomatic versus PSOA	JDM Asymptomatic versus PSOA	JDM Symptomatic versus Health	JDM Asymptomatic versus Health
	Mean	Range	Mean	Range	Mean	Range	Mean	Range					
Age	10.1 ± 4.7	3–18	10.8 ± 4.0	5–18	8.7 ± 4.0	3–17	10.6 ± 4.5	2–18	0.54	0.23	0.21	0.82	0.64
WBC	7.8 ± 3.5	3.7–15.2	7.0 ± 2.1	4.5–11	6.6 ± 1.9	3.6–13.1			0.36	0.09	0.40		
Hgb	12.7 ± 0.9	10.9–14.4	13.5 ± 1.0	11.7–15.9	12.8 ± 1.1	9.9–14.8			**0.007**	0.550	**0.03**		
Plat	300 ± 66	173–424	306 ± 84	151–422	326 ± 88	194–501			0.74	0.21	0.43		
Neu	4.6 ± 2.7	1.44–10.39	3.7 ± 1.8	1.71–9.72	3.4 ± 1.5	0.9–8.7			0.19	**0.03**	0.44		
Lym	2.0 ± 0.8	0.4–3.47	2.4 ± 1.0	0.85–5.06	2.4 ± 0.7	1.10–4.12			0.08	0.11	0.58		
Mon	0.9 ± 0.5	0.22–2.31	0.6 ± 0.2	0.25–1.3	0.6 ± 0.2	0.17–0.91			**0.03**	**0.002**	0.34		
ESR	17.0 ± 11.7	3–53	10.7 ± 10.1	1–41	8.8 ± 6.0	2–25			0.06	**0.001**	0.38		
CPK	1381 ± 3207	25–11332	87 ± 47	19–207	91 ± 30	56–162			0.07	0.12	0.80		
ALD	6.9 ± 5.8	2.6– >25	4.5 ± 1.7	2.5–8.5	4.8 ± 1.3	2.3–8.0			0.07	0.17	0.51		
LDH	327 ± 249	126–1236	199 ± 45	127–285	218 ± 46	123–319			**0.02**	**0.02**	0.15		
AST	68 ± 100	16–389	22.6 ± 7.2	12–35	26 ± 9	11–46			**0.04**	**0.02**	0.13		
ALT	43 ± 59	7–267	15.2 ± 6.1	9–35	19 ± 10	7–49			**0.03**	**0.03**	0.20		
CMAS	40.0 ± 13.6	0–52	51.6 ± 1.0	48–52	N/A				**0.0002**				

Gender of samples was as follows: JDM symptomatic, M10/F17; JDM asymptomatic, M4/F21; PSOA, M6/F25; healthy, M29/F14. Statistically significant p values (p < 0.05) are shown in bold.
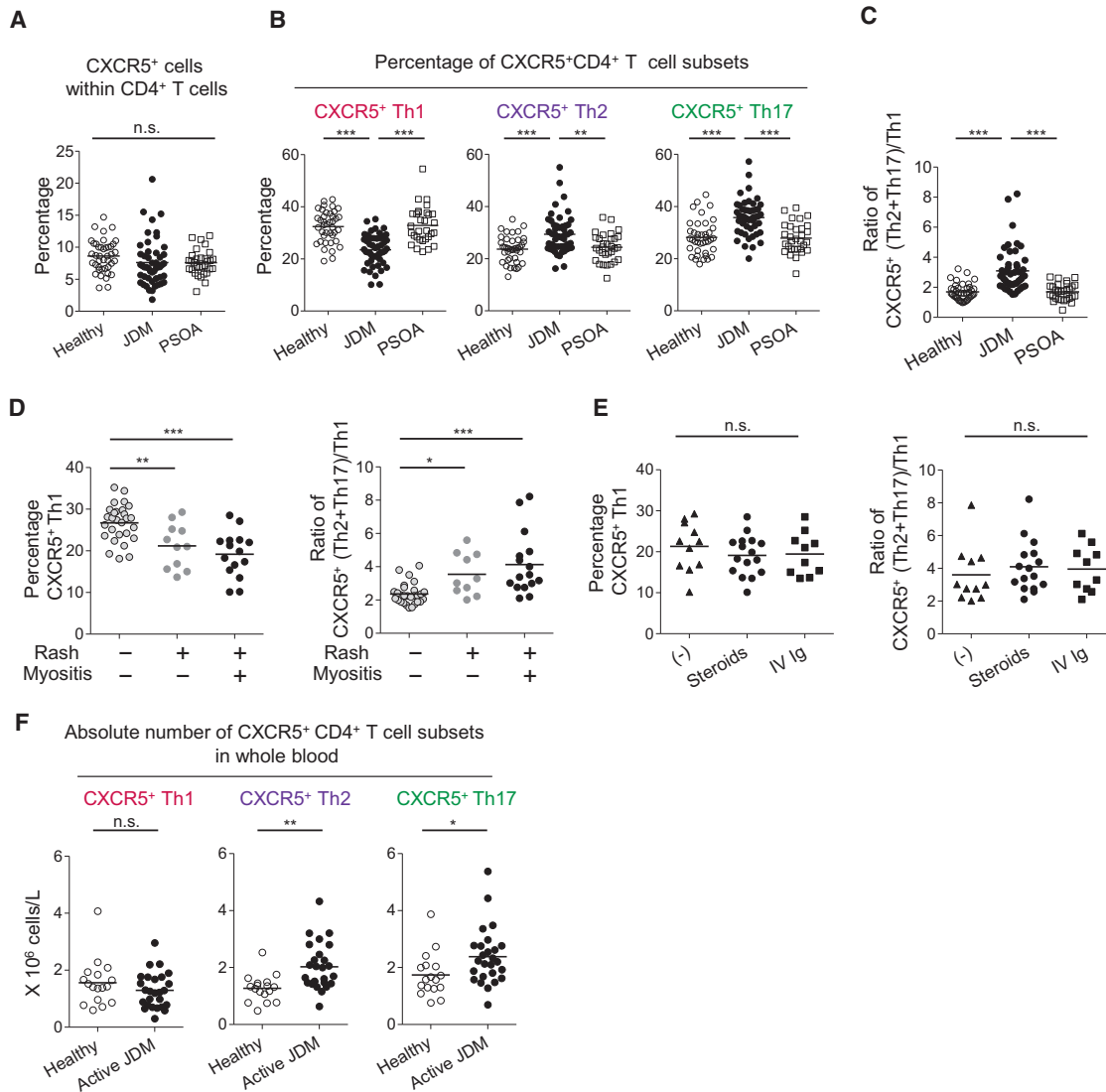
Figure 5. Blood CXCR5+ Th Cell Subsets Are Altered in JDM

(A) Percentage of CXCR5+ cells within CD4+ T cells in samples from JDM patients (n = 52), PSOA patients (n = 31), and age-matched healthy controls (n = 43).

(B) Percentage of each Th cell subset within blood CXCR5+CD4+ T cells. One-way ANOVA test, $p < 0.01$, $p < 0.001$.

(C) Ratio of CXCR5+ (Th2+Th17)/Th1 cells. One-way ANOVA test.

(D) Frequency of CXCR5+ Th1 cells and ratio of (Th2+Th17)/Th1 cells in JDM patients with different disease activities. One-way ANOVA test.

(E) Frequency of CXCR5+ Th1 cells and ratio of (Th2+Th17)/Th1 cells in active JDM patients receiving none, intravenous corticosteroids, or high-dose Ig treatments.

(F) The absolute cell numbers in blood were calculated based on the complete blood cell count, lymphocyte frequency within white blood cells, and the frequency of CXCR5+ Th cell subsets within the lymphocyte population. Student's t test.

t test; Th17: active JDM 2.4 ± 0.2 versus healthy 1.7 ± 0.2 × 10^6 cells/L; p = 0.03). Lastly, JDM patients displayed higher serum IgG concentrations than did PSOA and control groups (Figure S4C).

Analysis of blood B cell subsets revealed that JDM patients, particularly in those displaying both skin rash and muscular weakness, displayed higher numbers of circulating plasmablasts (CD19+CD20−CD27+CD38hi cells) (Arce et al., 2001) than PSOA patients and controls (Figures 6A and 6B). The number of circulating plasmablasts was similar among symptomatic patients regardless of treatment modality (Figure 6C). The fre-

quency of plasmablasts within CD19+ B cells correlated positively with the extent of skewing of CXCR5+ Th cell subsets toward Th2+Th17 cells (Figure 6D, top left) and negatively with the frequency of CXCR5+ Th1 cells (Figure 6D, top right). The correlation between the frequency of plasmablasts and the skewing of CXCR5+ Th cell subsets was limited to symptomatic patients (rash and/or muscular weakness) (Figure 6D, middle and bottom). In contrast, the frequency of plasmablasts did not correlate with the frequency of either total CXCR5+ CD4+ T cells (Figure 6E, left) or ICOS+CXCR5+CD4+ T cells (Figure 6E, right). Furthermore, neither the skewing of Th cell
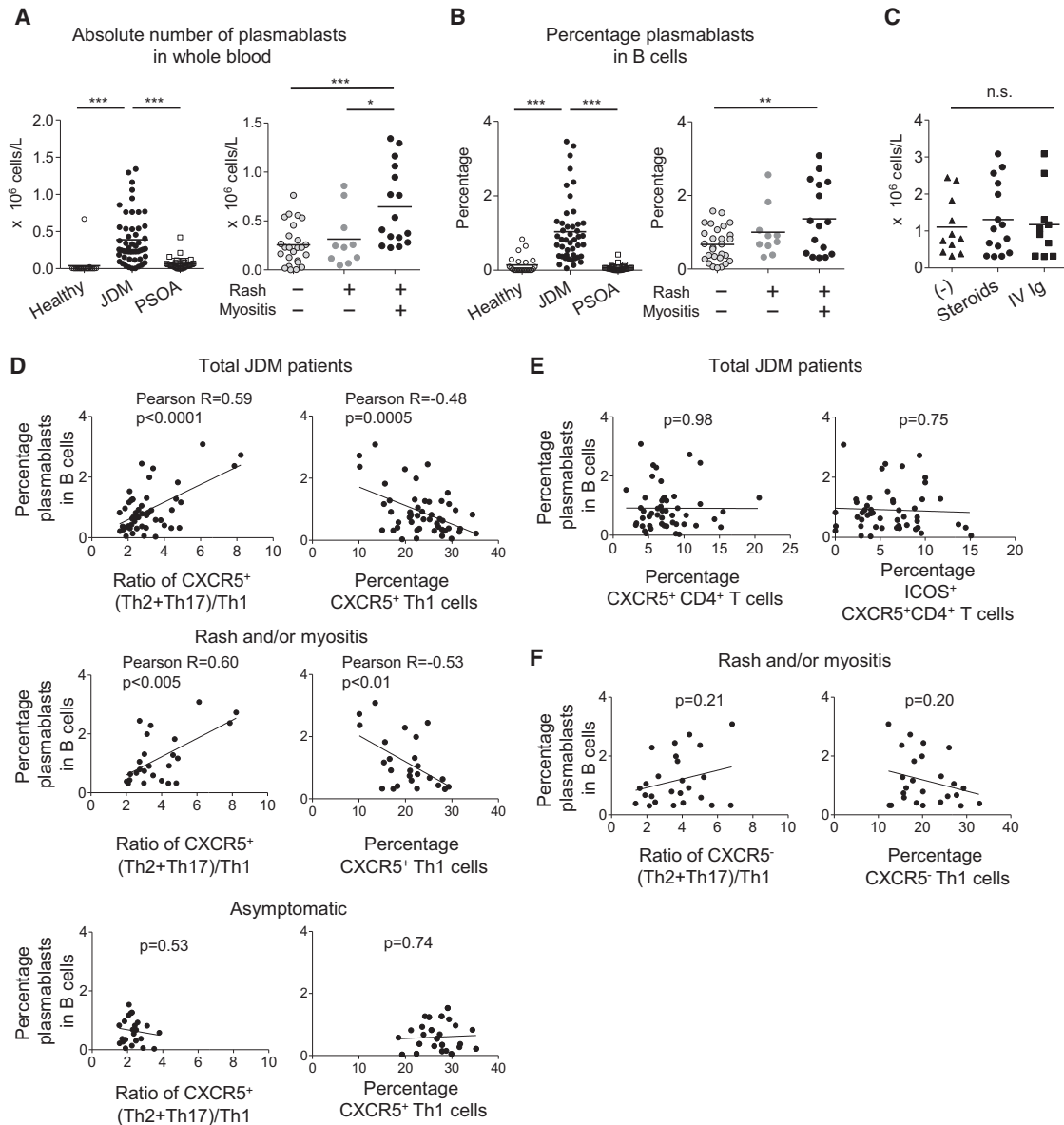
Figure 6. Skewing in Blood CXCR5+ Th Cell Subsets Correlates with B Cell Alteration

(A) The absolute number of plasmablasts in blood of JDM patients, PSOA patients, and healthy controls (left), and in JDM patients with different disease activities (right). One-way ANOVA test.

(B) Percentage of plasmablast within total CD19+ B cells.

(C) The absolute number of plasmablasts in blood of active JDM patients receiving none, intravenous corticosteroids, or high-dose Ig treatments.

(D) Correlation between the percentage of plasmablasts within CD19+ B cells and the ratio of CXCR5+ (Th2+Th17)/Th1 cells (left) or the frequency of CXCR5+ Th1 cells (right) in JDM. Pearson correlation coefficient and two-tailed p value are shown.

(E) Correlation between the percentage of plasmablasts within CD19+ B cells and the frequency of total CXCR5+CD4+ T cells (left) or of ICOS+ CXCR5+CD4+ T cells (right).

(F) Correlation between the percentage of plasmablasts and the ratio of CXCR5− (Th2+Th17)/Th1 (left) or the frequency of CXCR5− Th1 cells (right) in active JDM patients.

subsets within CXCR5−CD4+ T cells nor the frequency of CXCR5− Th1 cells correlated with the frequency of plasmablasts (Figure 6E).

Collectively, in JDM patients, the alteration of blood CXCR5+ Th cell subsets correlated with disease activity and with an increase in circulating plasmablasts.

DISCUSSION

Our study shows that blood CXCR5+CD4+ T cells share functional properties with Tfh cells from secondary lymphoid organs. In concordance with Tfh cells, blood CXCR5+CD4+ T cells induced naive and memory B cells to become Ig-producing cells

via IL-21, IL-10, and ICOS and secreted CXCL13. At variance with Tfh cells, blood CXCR5+CD4+ T cells barely expressed CD69 and ICOS, and PD-1 only at low intensity (Kim et al., 2001; Ma et al., 2009; Simpson et al., 2010), suggesting that they are in a resting state. Consistently, blood CXCR5+CD4+ T cells required cell activation to provide help to B cells through cognate interaction. Whereas CXCR5 can be expressed by any activated CD4+ T cells (Schaerli et al., 2001), dissociation in the expression of CXCR5 and activation molecules such as CD69 and ICOS suggests that blood CXCR5+CD4+ T cells do not represent recently activated cells. In contrast to Tfh cells, blood CXCR5+CD4+ T cells express CCR7 and CD62L, suggesting their capacity to migrate into secondary lymphoid organs. Thus, it is plausible that upon microbial invasion, CXCR5+CD4+ T cells draining into lymphoid organs interact with B cells presenting microbial antigens and induce their differentiation into Ig-producing cells or germinal center B cells through secretion of IL-21 (Linterman et al., 2010; MacLennan et al., 2003; Zotos et al., 2010).

Our study shows that human blood CXCR5+CD4+ T cells are composed of three subsets: Th1, Th2, and Th17 cells. CXCR5+ Th2 and CXCR5+ Th17 cells induced naive B cells to secrete Igs through IL-21 but differentially modulated isotype switch. Whereas CXCR5+ Th2 cells promoted IgG and IgE secretion, CXCR5+ Th17 cells promoted IgG and, in particular, IgA secretion. These findings suggest that Tfh cells associated with different Th cell subsets differentially shape the quality of human humoral immunity. In support of this hypothesis, only a fraction of tonsillar Tfh cells produces IL-4 together with IL-21 (Lane et al., 2005; Ma et al., 2009; Yu et al., 2009). Th2-type Tfh cells were also demonstrated in mice (King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). The difference between mouse and human Tfh cells might lie on the role of Th1-type cells. Mouse studies identified IFN-γ-secreting Th1-type Tfh cells in GCs, which promote the class-switching of GC B cells toward IgG2a (Reinhardt et al., 2009). This is in contrast to human CXCR5+ Th1 cells, which were incapable of helping B cells. Indeed, IFN-γ does not have any impact on isotype switching of human B cells (Banchereau et al., 1994).

Among blood CXCR5−CD4+ T cells, only CXCR5− Th2 cells induced naive B cells to become plasmablasts producing IgM and IgE. CXCR5− Th17 cells were unable to induce naive B cells to secrete Igs. Neither CXCR5− Th2 nor CXCR5− Th17 cells secreted IL-21 upon interaction with naive B cells. The molecular mechanisms whereby two different types of effectors, i.e., B helpers and non-B helpers, emerge from Th2 and Th17 cell subsets remain to be established. Multiple factors are probably involved in this process, including DC subsets that prime naive CD4+ T cells (Klechevsky et al., 2008), cytokines secreted by DCs (Deenick et al., 2010; Dienz et al., 2009; Schmitt et al., 2009) or other cell types including neighboring Th cells (Nurieva et al., 2008; Vogelzang et al., 2008), T cell receptor affinity against peptide-MHC complex (Fazilleau et al., 2009), and the interaction with B cells (Nurieva et al., 2008; Zaretsky et al., 2009).

Whether human blood CXCR5+CD4+ T cells originate from cells that migrated out of GCs or Tfh cell-committed extrafollicular helper cells (MacLennan et al., 2003; Poholek et al., 2010) will be challenging to address in humans. Notably, consistent with a previous report (Simpson et al., 2010), the expression of

BCL6 transcript was similar between blood CXCR5+ and CXCR5− Th cell subsets. However, the expression of *PRDM1* transcript was lower in all the Th cell subsets in CXCR5+ cells than their counterparts in CXCR5− cells. Given the reciprocal regulation between Bcl-6 and Blimp-1 in the generation of Tfh cells (Johnston et al., 2009), maintaining the expression of Blimp-1 at low amounts might be a feature of memory Tfh cells. Alternatively, in concordance with B cells (Kuo et al., 2007), downregulation of Bcl-6 might be necessary for germinal center Tfh cells to become memory cells.

Our study on JDM suggests that the alteration in the balance of Tfh cell subsets can be associated with autoimmunity in humans. Higher numbers of circulating plasmablasts in active JDM patients and a high relevance of antinuclear antibodies prompted us to analyze blood CXCR5+ subsets in these patients. Overrepresentation of Th2 and Th17 cells and underrepresentation of Th1 cells in both CXCR5+ and CXCR5− cells suggest that the overall regulation of Th cell differentiation is altered in JDM patients. The alteration in CXCR5+ Th cell subsets, however, correlated better with the frequency of circulating plasmablasts than that in CXCR5− Th cell subsets and thus represents a better biomarker to assess the dysregulation of B cell responses in this disease.

Our findings support the idea that humoral responses are differentially regulated by different subsets of Tfh cells in humans. This might have important implications for the design of novel vaccines. For example, induction of Th2- and Th17-, but not Th1-type, Tfh cells would be desired for efficient antibody responses to vaccination. In particular, the discovery of the Th17-type CXCR5+CD4+ T cell subset as a potent IgA inducer could guide the design of vaccines for protective mucosal immunity. Further characterization of blood CXCR5+CD4+ T cells as well as secondary lymphoid organ Tfh cells will provide insights into the pathogenesis and perhaps identify novel therapeutic targets for human autoimmune diseases.

EXPERIMENTAL PROCEDURES

Blood Samples

PBMCs purified from apheresis blood samples obtained from adult healthy volunteers were used in the experiments. Fresh blood samples were also collected from JDM (n = 52), PSOA patients (n = 31), and age-matched pediatric controls (n = 43). Detailed clinical characteristics, clinical lab data, and treatment at the time of analysis are shown in Table S1. The study was approved by the Institutional Review Boards (IRBs) of UT Southwestern Medical Center, Texas Scottish Rite Hospital, and Baylor Health Care System. Informed consent was obtained from parents or legal guardians.

Cell Isolation

CD4+ T cells enriched by negative selection were stained with the anti-CD4 FITC (RPA-T4), anti-CXCR5 PE (51505.111), anti-CD45RA TC (MEM-56), anti-CD14 APC (61D3), and anti-CD123 APC (AC145). Then, naive, CXCR5− memory, and CXCR5+ memory CD4+ T cells were sorted from APC-negative cell fractions. For CXCR5+ Th cell subset sorting, enriched CD4+CD45RA− T cells were stained with anti-CCR6 biotin (11A9) + SA-TC, anti-CXCR3 FITC (49801), anti-CXCR5 PE, and anti-CD4 APC. Positively selected CD19+ B cells were stained with anti-IgD FITC (IA6-2), anti-CD27 PE (L128), and anti-CD3 APC. Naive and memory B cells were sorted as IgD+CD27−CD3− and CD27+ CD3−CD19+ cells, respectively.

CD4+ T Cell and B Cell Coculture

Naive B cells were cocultured with sorted CD4+ T cells (2 × 10^4 cells each/well; 5 × 10^4 cells each/well for cytokine measurement) in the presence of

endotoxin-reduced SEB (1 μg/ml) in RPMI 1640 complete medium supplemented with 10% heat-inactivated FBS. Cytokine concentrations were measured in the culture supernatants at day 2 by Luminex, and the Ig concentrations were measured at day 12 by ELISA.

Real-Time RT-PCR
Total RNA was extracted from blood Th cell subpopulations or cultured B cells. Real-time PCR was set up with Roche Probes Master reagents and Universal Probe Library hydrolysis probes. PCR reaction was performed on the LightCycler 480 (Roche Applied Science) according to these conditions: step 1 (denaturation) at 95°C for 5 min, step 2 (amplification) at 60°C for 30 min, and step 3 (cooling) at 40°C for 30 s. The expression of each gene was normalized to housekeeping gene *ACTB*.

Phenotypical Analysis of Blood Samples
Whole blood cells were stained with these mAbs: CXCR5-Alexa488 (RF8B2), CCR6-PE (11A9), CD45RA-ECD (2H4LDH11LDB9), CXCR3-PECy5 (1C6), CD3-AF700 (UCHT1), CD4-Pacific Blue (RPA-T4), CD45-Pacific Orange (HI30), CD19-ECD (J3.119), CD20-PECy5 (2H7), CD38-PECy7 (HB7), and CD27-APCH7 (O323). The stained cells were analyzed with BD LSRII.

Statistics
The significance of the difference between groups was analyzed with one-way ANOVA test with Bonferroni correction. The significance of the difference between two groups was evaluated by F-test followed by the two-tailed Student's or paired t test. A Wilcoxon signed-ranks test was applied when data did not show Gaussian distribution. Pearson correlation coefficient and two-tailed p value were determined in the analysis of correlations.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at doi:10.1016/j.immuni.2010.12.012.

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