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Circulating Precursor $CCR7^{lo}PD\text{-}1^{hi}$ $CXCR5^{+}$ $CD4^{+}$ T Cells Indicate Tfh Cell Activity and Promote Antibody Responses upon Antigen Reexposure

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SUMMARY

Follicular B helper T (Tfh) cells support high affinity and long-term antibody responses. Here we found that within circulating $CXCR5^{+}$ $CD4^{+}$ T cells in humans and mice, the $CCR7^{lo}PD\text{-}1^{hi}$ subset has a partial Tfh effector phenotype, whereas $CCR7^{hi}$ $PD\text{-}1^{lo}$ cells have a resting phenotype. The circulating $CCR7^{lo}PD\text{-}1^{hi}$ subset was indicative of active Tfh differentiation in lymphoid organs and correlated with clinical indices in autoimmune diseases. Thus the $CCR7^{lo}PD\text{-}1^{hi}$ subset provides a biomarker to monitor protective antibody responses during infection or vaccination and pathogenic antibody responses in autoimmune diseases. Differentiation of both $CCR7^{hi}PD\text{-}1^{lo}$ and $CCR7^{lo}PD\text{-}1^{hi}$ subsets required ICOS and BCL6, but not SAP, suggesting that circulating $CXCR5^{+}$ helper T cells are primarily generated before germinal centers. Upon antigen reencounter, $CCR7^{lo}PD\text{-}1^{hi}$ $CXCR5^{+}$ precursors rapidly differentiate into mature Tfh cells to promote antibody responses. Therefore, circulating $CCR7^{lo}$ $PD\text{-}1^{hi}$ $CXCR5^{+}$ $CD4^{+}$ T cells are generated during active Tfh differentiation and represent a new mechanism of immunological early memory.

INTRODUCTION

Previous studies performed by ourselves and others independently characterized a subset of helper T cells, termed follicular B helper T (Tfh) cells, with a distinct gene expression profile and a specialized role in facilitating antibody responses (Breitfeld et al., 2000; Chtanova et al., 2004; Crotty, 2011; Fazilleau et al., 2009; Kim et al., 2004; Kim et al., 2001; King et al., 2008; Nurieva et al., 2008; Schaerli et al., 2000; Yu et al., 2009a; Yu and Vinuesa, 2010a). Without Tfh cells, protective antibody responses are largely diminished, as evidenced by recurrent infections in patients with primary immunodeficiency due to mutations in inducible costimulator (ICOS) or the signaling adaptor SLAM-associated protein (SAP), two key molecules for Tfh cell differentiation (King et al., 2008). Although Tfh cells are essential for protective antibody responses, excessive or dysregulated Tfh cells can also result in the generation of autoantibodies and, subsequently, autoimmune diseases (Craft, 2012; Yu and Vinuesa, 2010b).

The fundamental role of Tfh cells in humoral immunity has called forth many studies to understand their role in human infections (Cubas et al., 2013; Feng et al., 2011; Lindqvist et al., 2012; Pallikkuth et al., 2012), autoimmune diseases (Morita et al., 2011; Simpson et al., 2010; Zhu et al., 2012), and vaccination (Bentebibel et al., 2013; Pallikkuth et al., 2012). These studies mainly focused on human blood samples and clearly showed a positive correlation between the circulating CXC

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chemokine receptor 5^{+} (CXCR5^{+}) CD4^{+} T cells and either protective antibodies or autoantibodies (Bentebibel et al., 2013; Morita et al., 2011; Pallikkuth et al., 2012; Simpson et al., 2010; Zhu et al., 2012). Moreover, circulating CXCR5^{+} T cells could be further distinguished according to their coexpression of T helper 1 (Th1), Th2, or Th17 features that possess distinct capabilities to support B cell antibody production (Bentebibel et al., 2013; Morita et al., 2011).

Although there is a consensus that circulating CXCR5^{+} CD4^{+} T cells are closely associated with Tfh cells, their ontogeny is still poorly defined. Also unclear is the important question of whether circulating cells relate to ongoing or past Tfh responses (Craft, 2012; Pepper and Jenkins, 2011; Yu and Vinuesa, 2010a). There is still a lack of a reliable method to identify active Tfh differentiation. The absence of information on the ontogeny of circulating CXCR5^{+} CD4^{+} T cells also raises the controversy over the existence of bona fide Tfh cells circulating in blood.

Here, we show that circulating CXCR5^{+} helper T cells comprise CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} subsets. In human blood, CCR7^{lo}PD-1^{hi} CXCR5^{+} helper T cells were transiently increased after vaccination. Circulating CCR7^{lo}PD-1^{hi} CXCR5^{+} helper T cells in mice phenocopy their human counterpart and follow nearly identical kinetics during antibody responses. The generation of both CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} CXCR5^{+} helper T cells is dependent on the costimulatory receptor ICOS and the transcription factor B cell lymphoma 6 (BCL6), but not SAP, suggesting that circulating CXCR5^{+} helper T cells are primarily generated before entering germinal center (GC). In in vitro T-B coculture, circulating CCR7^{lo}PD-1^{hi} CXCR5^{+} helper T cells were the most potent among CD4^{+} T cell subsets to induce plamablast or plasma cell differentiation and antibody production. Upon antigen reexposure in vivo, circulating CCR7^{lo}PD-1^{hi} CXCR5^{+} helper cells rapidly differentiated into mature Tfh cells to support GC formation, suggesting their capability as early memory cells to promote antibody responses. Together, these findings provide new insights into the ontogeny of circulating CXCR5^{+} helper T cells. They reveal that the Tfh precursor CCR7^{lo}PD-1^{hi} CXCR5^{+} cells in blood correlates with active Tfh differentiation in secondary lymphoid organs, and there is little evidence for bona fide Tfh cells in blood. Accordingly, in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients, increased levels of the CCR7^{lo}PD-1^{hi} subset correlated with elevated autoantibody profiles and more severe disease activities.

RESULTS

Circulating Antigen-Experienced CXCR5^{+} CD4^{+} Helper T Cells Are Heterogeneous

To understand the ontogeny of circulating CXCR5^{+} helper T cells, this population was investigated more closely. All CXCR5^{+} CD4^{+} T cells have downregulated CD45RA (see Figure S1A available online) as previously reported (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000; Simpson et al., 2010), reflecting an antigen-experienced phenotype. Human circulating effector and memory cells contain discrete populations of central memory (Tcm) and effector memory (Tem) T cells, which are characterized by distinct homing capability and effector functions (Sallusto et al., 2004). Tcm cells constitutively express CCR7, whereas Tem cells do not (Sallusto et al., 2004). The majority of circulating CXCR5^{+} CD4^{+} T cells express CCR7 (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000). However, in contrast to naive CD4^{+} T cells, which uniformly expressed high amounts of CCR7, some of circulating CXCR5^{+} CD4^{+} T cells expressed low amounts of CCR7 (Figure S1B), suggesting heterogeneity in circulating CXCR5^{+} helper T cells. When CCR7 and programmed cell death 1 (PD-1), a marker for recent antigen exposure (Agata et al., 1996) and Tfh cells (Crotty, 2011; Haynes et al., 2007; King et al., 2008; Yu and Vinuesa, 2010a), were combined, CXCR5^{+} helper T cells could be further segregated into two populations: CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi}. There was an expansion of the CCR7^{lo}PD-1^{hi} population (1.80% ± 1.30%, $p = 0.015$) in individuals 1 week after influenza vaccination (Figure 1A; Figure 1B).

As in human blood, CCR7^{hi}PD-1^{lo} cells represent the majority of CXCR5^{+} T cells in mouse blood under resting conditions (Figure 1C). When mice were intraperitoneally (i.p.) immunized with sheep red blood cells (SRBCs), there was a transient increase of the CCR7^{lo}PD-1^{hi} subset 1 week after immunization (Figure 1C; Figure 1D). In contrast, the CCR7^{hi}PD-1^{lo} subset in total CD4^{+} T cells remained unchanged at day 7 but increased at day 14 after the peak of the active differentiation of Tfh cells in the lymphoid organs (Figure 1C; Figure 1E).

To track antigen-specific cells, naive CD45.2 OT-II CD4^{+} T cells were transferred into congenic CD45.1 mice immunized i.p. with ovalbumin (OVA) in alum. Seven days after immunization, CXCR5^{+} OT-II cells in blood were predominantly CCR7^{lo}PD-1^{hi} (Figure 1F; Figure 1G). However, the phenotype of circulating CXCR5^{+} OT-II cells gradually drifted and 3 weeks after immunization, CXCR5^{+} OT-II cells in blood were essentially CCR7^{hi}PD-1^{lo} (Figure 1F; Figure 1H). Therefore, in both mouse and humans, circulating CXCR5^{+} helper T cells are heterogeneous with different phenotypic subsets demonstrating distinct differentiation kinetics during immune response.

Distinct Phenotype of Circulating CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} Subsets

Phenotypic analysis of naive CD4^{+} T cells, the circulating CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} subsets, as well as Tfh cells in lymphoid organs, displayed a similar pattern of expression of all proteins examined between human and mouse cells (Figure 2). Tfh cells have been reported to express highest amounts of CXCR5, PD-1, and ICOS and low amounts of CCR7 (Crotty, 2011). CD57 and GL7 are suggested markers for GC Tfh in humans (Kim et al., 2001; King et al., 2008) and mice (Yusuf et al., 2010), respectively. The CCR7^{lo}PD-1^{hi} subset of circulating CXCR5^{+} CD4^{+} T cells in blood expressed all these surface markers, albeit at intermediate levels (Figure 2A; Figure S2C). Tfh cells express high amounts of the key Tfh transcription factor BCL6 (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009b), as well as SAP, which is indispensible for stable T-B interaction during Tfh cell differentiation (Cannons et al., 2011; Qi et al., 2008). BCL6 was not expressed by the CCR7^{lo}PD-1^{hi} subset, although SAP expression in these cells was higher than in naive cells (Figure 2B; Figure S2D). In contrast, the CCR7^{hi}PD-1^{lo} subset of circulating CXCR5^{+} CD4^{+} T cells

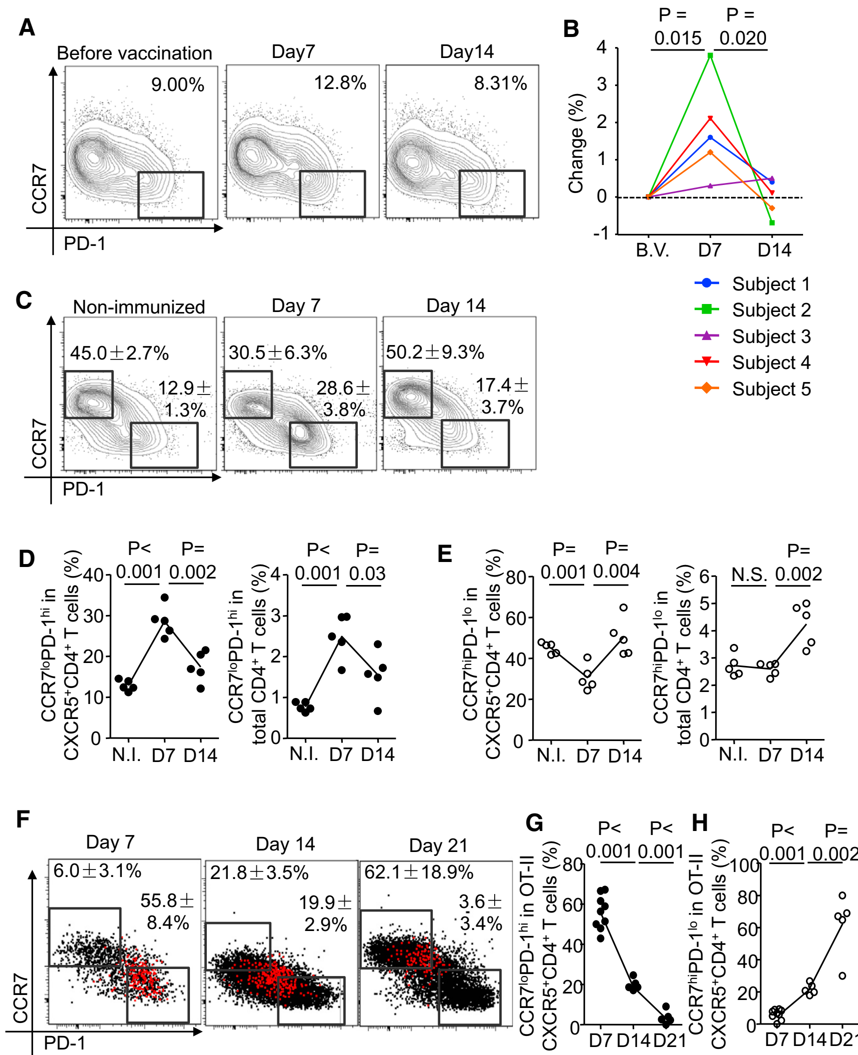

Figure 1. Circulating CXCR5⁺ CD4⁺ T Cells Are Heterogeneous and Are Composed of CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} Subsets

(A) PBMCs from healthy individuals 1 or 2 weeks after seasonal influenza vaccination were analyzed. FACS plots show CCR7 and PD-1 expression on CXCR5^{+}CD45RA^{-} CD4^{+} T cells from a representative individual.

(B) Statistics (n = 5) show the change in percentage of CCR7^{lo}PD-1^{hi} within the CXCR5^{+} CD4^{+} T cells at indicated time points compared to those before vaccinations (B.V.).

(C–E) Mice were immunized i.p. with SRBCs and the blood was analyzed at indicated points. FACS plots show CCR7 and PD-1 expression on CXCR5^{+} CD4^{+} T cells.

(F–H) CD45.2 naive OT-II cells were i.v. injected into CD45.1 mice followed by i.p. immunization with OVA in alum. Blood was analyzed at indicated time points after immunization. FACS plots show CCR7 and PD-1 expression on CXCR5^{+} CD4^{+} T cells (black represents recipient CD45.1 cells; red represents donor CD45.2 OT-II cells; statistics shown for transferred OT-II cells). Data are presented as mean ± SD. Statistics (n = 5–9) show the results of individual mice and the average values of each group are linked by the trend line. N.S., not significant. See also Figure S1.

expressed none of these Tfh markers (Figures 2A and 2B; Figures S2C and S2D). Similar to naive CD4^{+} T cells, CD62L and IL-7R were highly expressed in this subset, whereas the proliferation marker Ki-67 was expressed in low amounts (Figure 2C; Figure S2E).

Cytokines are the major effector molecules of T helper cells, and their expression was examined in CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} subsets in either CXCR5^{+}CD45RA^{-} or CXCR5^{-}CD45RA^{-} CD4^{+} T cells in blood 1 week after influenza vaccination. Interferon-γ (IFN-γ), interleukin-4 (IL-4), and IL-17, the representative cytokines for Th1, Th2, and Th17 cells, were predominantly expressed by the CCR7^{lo}PD-1^{hi} subset in CXCR5^{-} cells (Figure 2D). The CCR7^{lo}PD-1^{hi} subset in CXCR5^{+} cells expressed small amounts of IL-17 and IL-4 and large amounts of IL-21, a key cytokine secreted by Tfh cells to support GC responses (Linterman et al., 2010; Nurieva et al., 2008; Vogelzang et al., 2008; Zotos et al., 2010) (Figure 2E). This subset also expressed notable amounts of IFN-γ (Figure 2D). Cytokine expression in the CCR7^{hi}PD-1^{lo} subset either within the CXCR5^{+} or CXCR5^{-} population was limited (Figure 2D; Figure S2E). Thus the CCR7^{lo}PD-1^{hi} subset in the circulating CXCR5^{+} CD4^{+} T cells possessed a partial Tfh phenotype as determined by both surface and intracellular markers and cytokine profiles, whereas the CCR7^{hi}PD-1^{lo} subset showed a resting status similar to Tcm cells.

Human blood CXCR5^{+} CD4^{+} T cells can be divided into different functional subsets based on the expression of chemokine receptors CXCR3 and CCR6 (Bentebibel et al., 2013; Morita et al., 2011). CXCR3 and CCR6 expression were therefore examined in blood 1 week after influenza vaccination. As reported previously (Morita et al., 2011), the expression of CXCR3 and CCR6 in individual subsets largely correlated with the expression of IFN-γ and IL-17, respectively (Figure 2D; Figure S2F). IFN-γ and IL-17 were highly expressed by the CCR7^{lo}PD-1^{hi} subset in CXCR5^{-}CD45RA^{-} cells (Figure 2D) and so were CXCR3 and CCR6 (Figure 2F). Notably, high CCR6 but low IL-17 expression was found in the CCR7^{hi}PD-1^{lo} subset in CXCR5^{+}CD45RA^{-} cells (Figure 2F).

We next asked what drives expression of chemokine receptors on circulating CD4^{+} T cells and whether the pattern of chemokine receptors correlates with Tfh differentiation. Naive CD45.2 OT-II CD4^{+} T cells were transferred into congenic CD45.1 mice immunized i.p. with OVA in either alum or Sigma Adjuvant System (SAS). Alum induced a Th2-type response, whereas SAS induced a Th1-type response, as indicated by high expression of IFN-γ and CXCR3 in transferred OT-II cells in spleens (Figures S2F and S2G). There was a similar Tfh

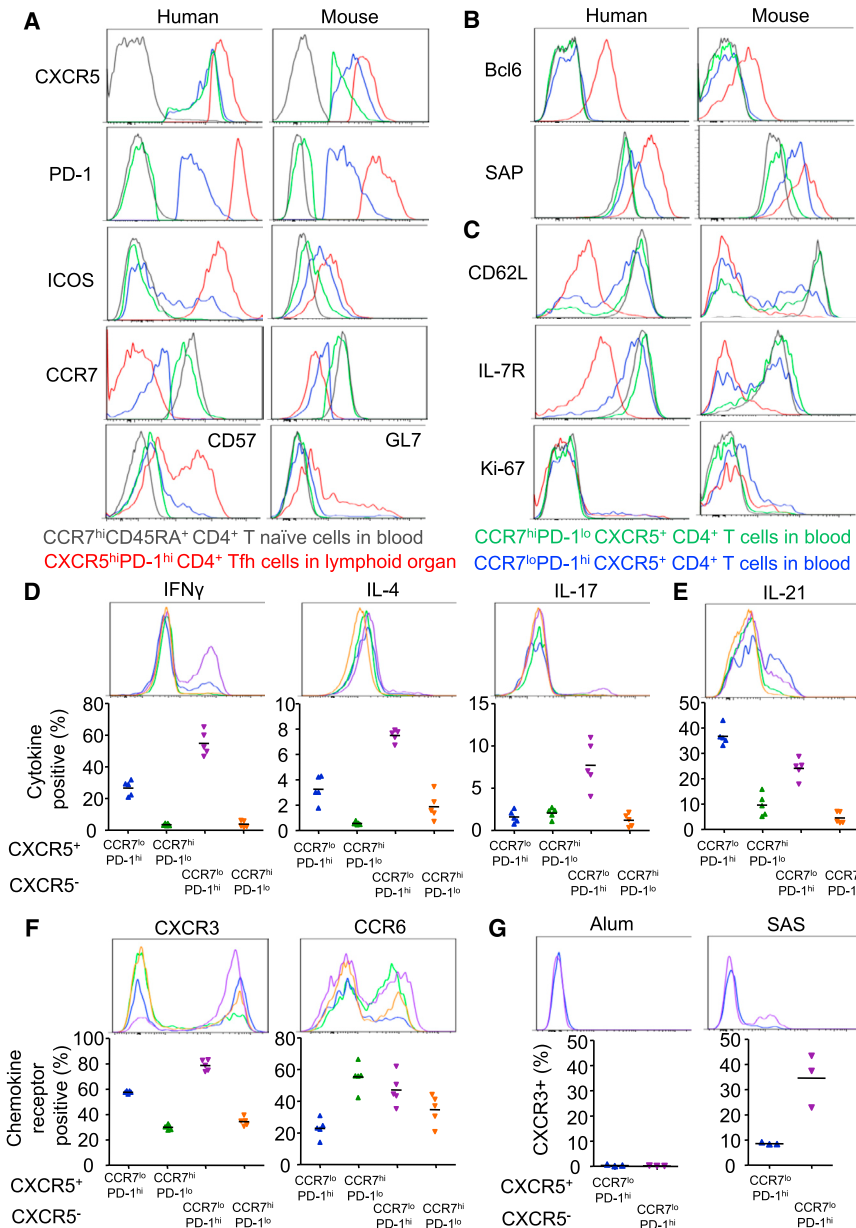

Figure 2. Distinct Phenotype of CCR7^{hi} PD-1^{lo} and CCR7^{lo}PD-1^{hi} Subsets

(A–C) Representative histograms and statistics (n = 5) show the expression of indicated surface molecules in individual populations as per gating in Figures S2A and S2B.

(D–F) Representative histograms and statistics (n = 5) showing cytokine and chemokine receptor expression in indicated populations of PBMCs from individuals after influenza vaccination. Statistics show the results of individuals and the average values of each group.

(G) CD45.2 naive OT-II cells were i.v. injected into CD45.1 mice followed by i.p. immunization with OVA in alum or Sigma Adjuvant System (SAS). Representative histograms and statistics (n = 5) show CXCR3 expression in indicated populations of PBMCs 7 days after immunization. See also Figure S2.

differentiation between the two types of adjuvant (Figure S2H). Corresponding to the response in the spleens, circulating OT-II cells in the blood also expressed higher amounts of CXCR3 in both CXCR5^{+} and CXCR5^{-} subsets in response to SAS compared to alum (Figure 2G). Therefore, chemokine receptors per se reflect the polarization of the immune response for Tfh differentiation rather than active Tfh cell differentiation.

Differentiation of Circulating Antigen-Experienced CXCR5^{+} CD4^{+} T Cells Is Dependent on Tfh Pathways

It has been previously reported that the circulating CXCR5^{+} CD4^{+} T cells are greatly reduced in ICOS-deficient patients (Bossaller et al., 2006) and that CXCR5 upregulation is impaired on CD4^{+} T cells in BCL6-deficient mice (Yu et al., 2009b). To examine whether the Tfh differentiation pathway intrinsically instructs circulating CXCR5^{+} CD4^{+} T cells, we transferred naive CD45.2 OT-II CD4^{+} T cells deficient in either ICOS or BCL6 into congenic CD45.1 *Cd28*$^{-/-}$ mice immunized i.p. with OVA in alum. Seven days after immunization, all transferred cells had been stimulated by antigen and undergone proliferation, indicated by high expression of CD44 and diluted carboxyfluorescein succinimidyl ester (data not shown). In mice that received wild-type (WT) OT-II cells, about a quarter of the antigen-specific circulating OT-II cells expressed CXCR5 (Figure 3A). These CXCR5^{+} antigen-experienced cells accounted for approximately 2% of total CD4^{+} T cells (Figure 3B). Transferred T cells that were deficient in BCL6 or ICOS were unable to differentiate into Tfh cells or support GC formation in spleen (Figures S3A and S3B). Similarly, *Bcl6*$^{-/-}$ or *Icos*$^{-/-}$ T cells were incapable of differentiating into CXCR5^{+} cells in blood (Figure 3A; Figure S3B) although they were able to differentiate into CXCR5^{-} cells, comparable to WT cells (Figure 3C).

Circulating Antigen-Experienced CXCR5^{+} CD4^{+} T Cells Are Generated Primarily prior to GC Reaction

The ICOS-mediated upregulation of BCL6 and CXCR5 and the subsequent initiation of Tfh differentiation can occur as early as the second cell division during the interaction of antigen-specific T cells with dendritic cells (Baumjohann et al., 2011; Choi et al., 2011; Kerfoot et al., 2011). CXCR5^{+} CD4^{+} T cells might enter the circulation during initial Tfh differentiation, prior to GC formation or from Tfh cells recently exiting from GC responses, as suggested by the observation that weeks after immunization, Tfh cells can downregulate BCL6 expression and gain the potential to become memory cells (Kitano et al., 2011). When does

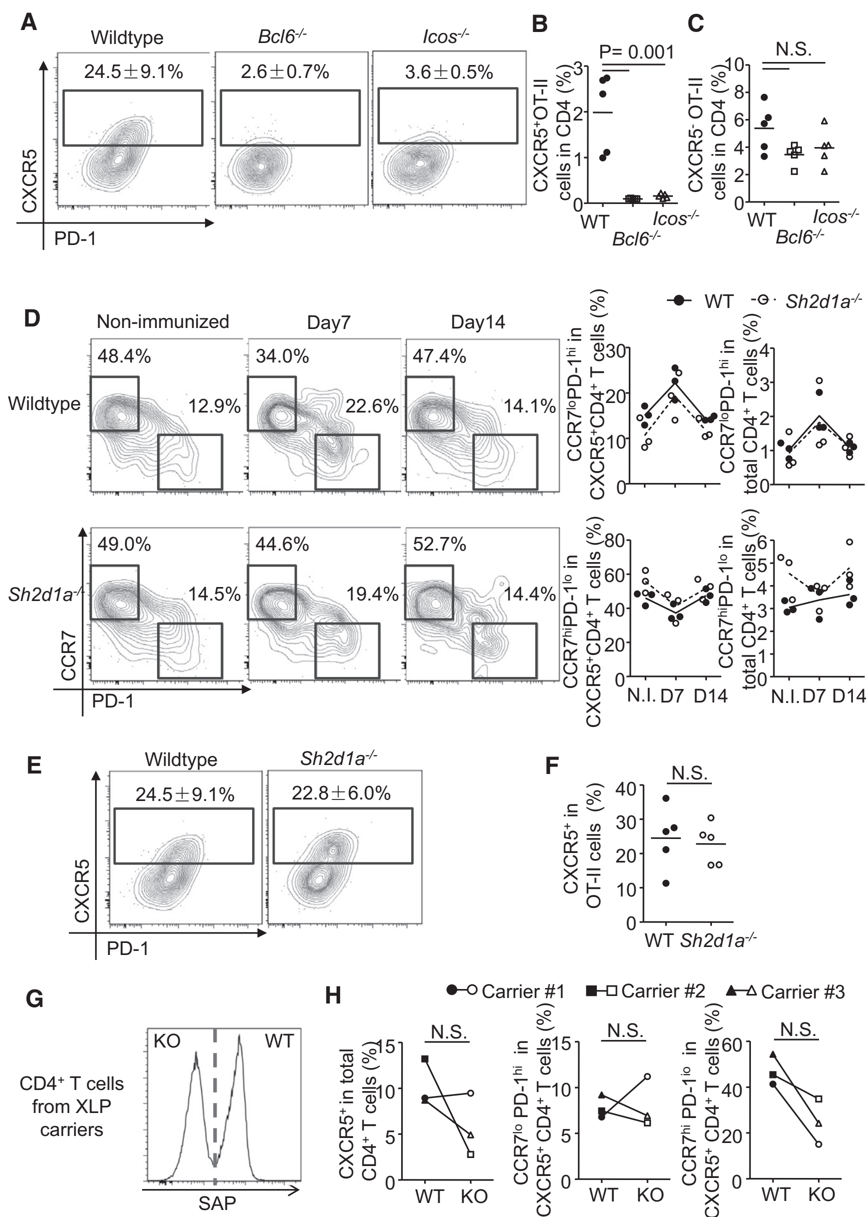

Figure 3. Both CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} Subsets of CXCR5^{+} CD4^{+} T Cells in Blood Are Primarily Generated before GCs

CD45.2 naive OT-II WT or BCL6- or ICOS-deficient cells were i.v. injected into CD45.1 *Cd28*$^{-/-}$ mice followed by i.p. immunization with OVA in alum. Blood was analyzed 7 days after immunization. Representative FACS plots show CXCR5 and PD-1 expression on CD4^{+} T cells (A).

Statistical analysis (n = 5) of data from individual mice and the average value of each group (B and C).

(D) WT or SAP-deficient mice were immunized with SRBCs and blood was analyzed at indicated time points. FACS plots show CCR7 and PD-1 on CXCR5^{+} CD4^{+} T cell. Statistics (n = 3) shows the results of individual mice and the average values of each group are linked by the trend line. CD45.2 naive OT-II WT or SAP-deficient cells were i.v. injected into CD45.1 *Cd28*$^{-/-}$ mice followed by i.p. immunization with OVA in alum. Blood was analyzed 7 days after immunization. Representative FACS plots show CXCR5 and PD-1 expression on CD4^{+} T cells (E).

Statistical analysis (n = 5) of data from individual mice and the average value of each group (F).

PBMCs from XLP carriers (n = 3) were examined by FACS. SAP-sufficient or -deficient cells were identified by intracellular staining (G).

Paired analysis between SAP-sufficient or -deficient cells (H). See also Figure S3.

circulating antigen-experienced CXCR5^{+} CD4^{+} T cell differentiation deviate from Tfh differentiation (Craft, 2012; Yu and Vinuesa, 2010a)? To address this question, an ideal approach would be to measure the circulating CXCR5^{+} CD4^{+} T cells in a scenario that allows steps of early Tfh differentiation but not terminal Tfh differentiation or GC formation. This scenario occurs in *Sh2d1a*$^{-/-}$ mice. Without SAP, Tfh differentiation and GC formation are severely impaired despite the upregulation of ICOS, BCL6, and CXCR5 (Choi et al., 2011; Deenick et al., 2010).

When SAP-deficient mice were immunized i.p. with SRBCs, both CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} subsets of circulating CXCR5^{+} CD4^{+} T cells were formed with kinetics similar to that of WT mice (Figure 3D). When transferred into *Cd28*$^{-/-}$ mice immunized i.p. with OVA in alum, SAP-deficient OT-II cells were able to generate circulating CXCR5^{+} cells in blood comparable to those from WT OT-II cells (Figure 3E; Figure S3F), although few Tfh cells or GCs formed in the spleens (Figures S3A and S3B). Sublethally irradiated mice were reconstituted with mixed bone marrow from WT and *Sh2d1a*$^{-/-}$ mice in a 50%:50% ratio, and the generation of circulating CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} subsets was examined in this competitive environment. If mature Tfh cells that have exited from GC response contribute significantly to circulating CXCR5^{+} CD4^{+} T cells, fewer SAP-deficient circulating CXCR5^{+} CD4^{+} T cells would be observed. However, both circulating CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} subsets were present in similar percentages between WT and *Sh2d1a*$^{-/-}$ CD4^{+} T cells before (Figure S3C) and after immunization with SRBCs (Figure S3D).

To test whether the same mechanism also operates in humans, we examined blood from patients diagnosed with X-linked lymphoproliferative disease (XLP). Due to mutations in the *SH2D1A* gene, T cells from these patients lack SAP expression (Cannons et al., 2011). The percentages of circulating CXCR5^{+} CD4^{+} T cells and the CCR7^{hi}PD-1^{lo} and CCR7^{lo}PD-1^{hi} subsets in two XLP subjects fell within the normal ranges of healthy controls (Figures S3E and S3F). The very rare frequency of XLP patients prevents the examination of a larger cohort. We then examined female XLP carriers who, due to random X-inactivation,

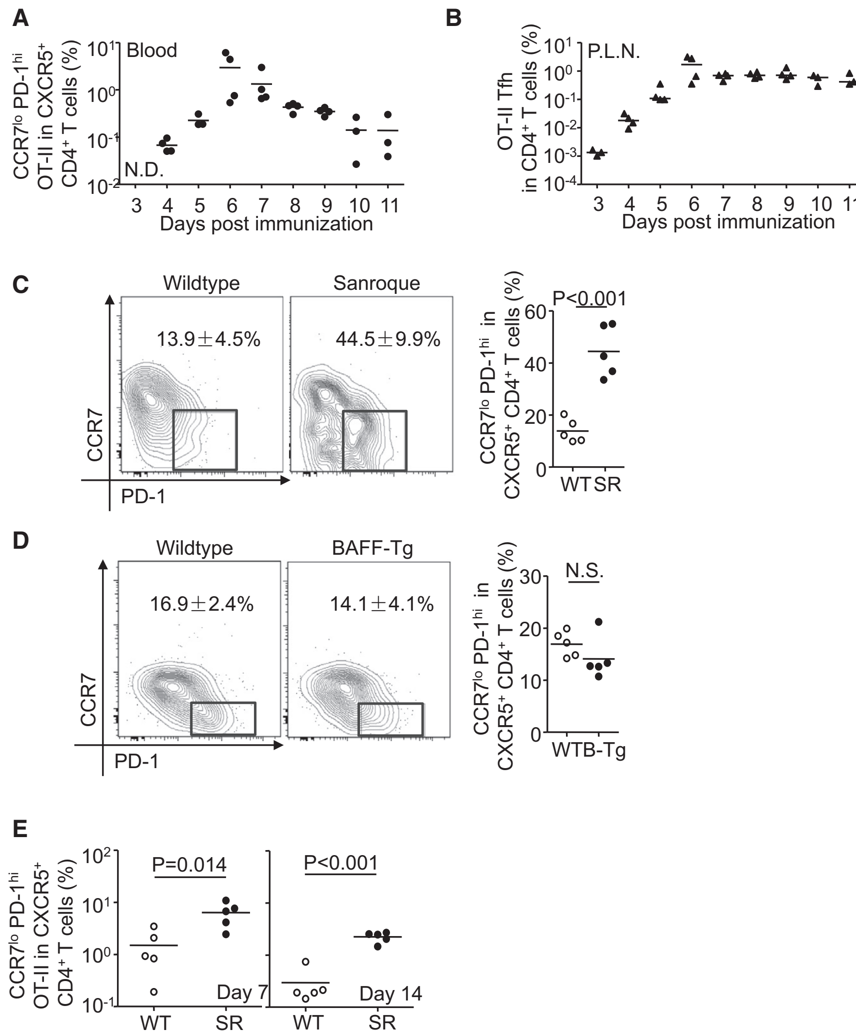

Figure 4. Increased Percentages of Circulating $CCR7^{lo}PD\text{-}1^{hi}$ $CXCR5^{+}$ $CD4^{+}$ T Cells Correlates with an Active Tfh Program

CD45.2 naive OT-II cells were i.v. injected into CD45.1 mice followed by s.c. immunization with OVA in alum. Blood (A) and popliteal lymph nodes (PLN) (B) were analyzed from 3 to 11 days after immunization. Dot plots show the numbers of indicated populations at each time points. FACS plots show CCR7 and PD-1 expression on $CXCR5^{+}$ $CD4^{+}$ T cells in blood from 8- to 12-week-old *sanroque* or WT mice (C) or from 15- to 20-week-old BAFF-Tg or WT mice (D).

(E) CD45.2 naive OT-II cells from *sanroque* (SR) or WT mice were i.v. injected into CD45.1 mice then immunized i.p. with OVA in alum. Blood was analyzed at indicated time points after immunization. Statistics (n = 5) shows the results of individual mice with average values. See also Figure S4.

contain both SAP-sufficient and SAP-deficient cells (Figure 3G). Between SAP-sufficient and -deficient $CD4^{+}$ T cells within individual carriers, there was no significant difference in total circulating $CXCR5^{+}$ $CD4^{+}$ T cells, neither in $CCR7^{hi}PD\text{-}1^{lo}$ or $CCR7^{lo}PD\text{-}1^{hi}$ subsets, although there was a trend of decline of the $CCR7^{hi}PD\text{-}1^{lo}$ subset (Figure 3H). These results support a model whereby in both mice and humans, $CCR7^{hi}PD\text{-}1^{lo}$ and $CCR7^{lo}PD\text{-}1^{hi}$ subsets in the circulating $CXCR5^{+}$ $CD4^{+}$ T cells are primarily generated before T cells differentiate into mature Tfh effector cells and participate in GC responses.

Increased Circulating $CCR7^{lo}PD\text{-}1^{hi}$ $CXCR5^{+}$ $CD4^{+}$ T Cells Correlate with Active Tfh Program

The occurrence of the $CCR7^{lo}PD\text{-}1^{hi}$ $CXCR5^{+}$ $CD4^{+}$ T cells in blood correlates with active Tfh cell differentiation in secondary lymphoid organs (Figure 1) and they share many molecular requirements for differentiation (Figure 3). We then asked whether the $CCR7^{lo}PD\text{-}1^{hi}$ subset in blood could be utilized as a qualitative or quantitative measurement of Tfh cell differentiation in secondary lymphoid organs.

In order to track the kinetics of the differentiation of antigen-specific $CCR7^{lo}PD\text{-}1^{hi}$ $CXCR5^{+}$ $CD4^{+}$ T cells in blood, CD45.2 naive OT-II $CD4^{+}$ T cells were transferred into CD45.1 congenic mice immunized subcutaneously (s.c.) with OVA in alum. $CCR7^{lo}PD\text{-}1^{hi}CXCR5^{+}$ OT-II cells became detectable in the blood 4 days after immunization, peaked at days 6 and 7, and then declined (Figure 4A). OT-II Tfh cells in the draining popliteal lymph nodes also peaked at days 6 and 7 after immunization and numbers were maintained (Figure 4B). These results clearly demonstrate a strong correlation between the high percentages of the $CCR7^{lo}PD\text{-}1^{hi}$ subset in blood and active Tfh cell differentiation (day 3–7) in the draining secondary lymphoid organs.

We then examined the correlation between the high percentage of $CCR7^{lo}PD\text{-}1^{hi}$ cells among circulating $CXCR5^{+}$ $CD4^{+}$ T cells and active Tfh cell differentiation in the secondary lymphoid organs of *sanroque* (SR, $Roquin^{san/san}$) mice, a model for systemic autoimmunity driven by aberrant differentiation of Tfh cells (Linterman et al., 2009; Vinuesa et al., 2005; Yu et al., 2007). In *sanroque* mice, a striking 3-fold increase in the percentage of $CCR7^{lo}PD\text{-}1^{hi}$ cells among total $CXCR5^{+}$ $CD4^{+}$ T cells in blood was observed (Figure 4C). The increase in $CCR7^{lo}PD\text{-}1^{hi}$ cell percentages correlated with the amount of autoantibodies against double-stranded DNA (dsDNA) (Figure S4).

The increase in percentages of circulating $CCR7^{lo}PD\text{-}1^{hi}$ subset in *sanroque* mice could be explained by either Tfh differentiation stimulated by autoimmune-induced self-antigen exposure or an intrinsic bias of $CD4^{+}$ T cells to differentiate to a Tfh fate. To address the first possibility, we used another model of systemic autoimmunity, in which a B cell-activating factor (BAFF) transgene (Tg) drives pathology in a T cell-independent manner (Mackay and Schneider, 2009). Percentages of $CCR7^{lo}PD\text{-}1^{hi}$ cells were not increased in the blood of BAFF-Tg mice (Figure 4D), despite the presence of self-antigen, autoantibodies, and other autoimmune indicators (Mackay and Schneider,

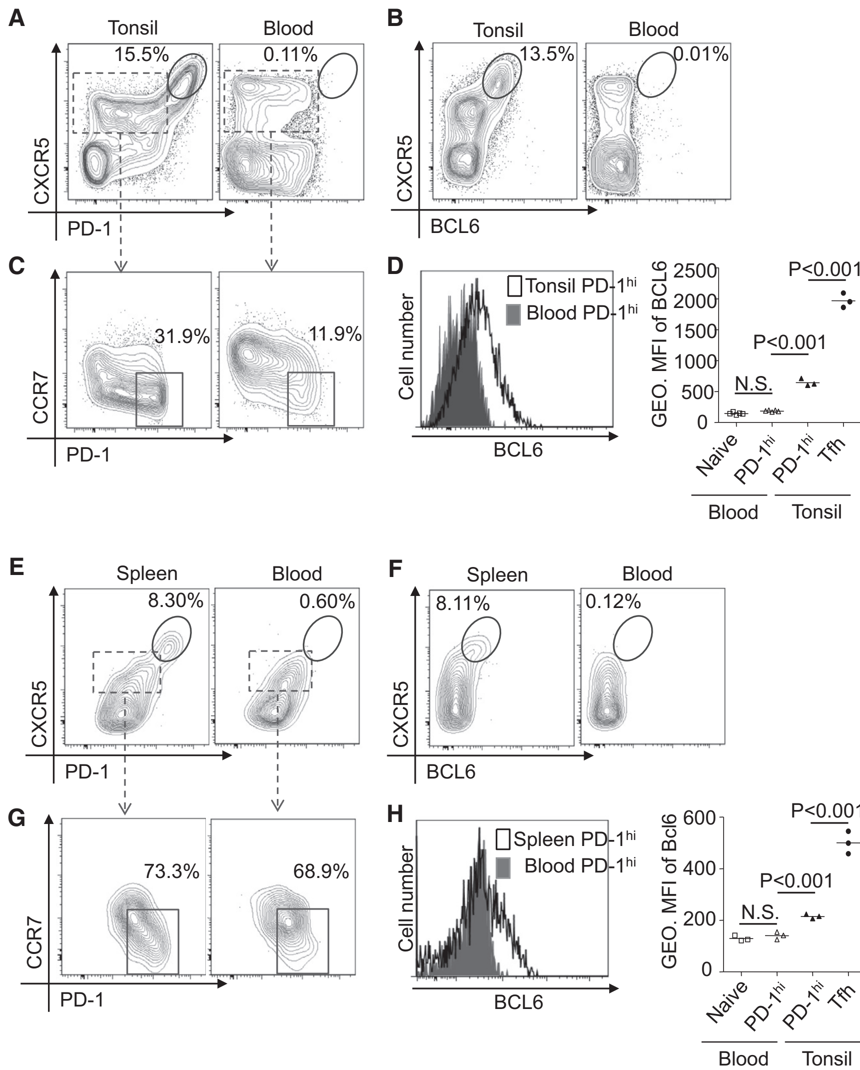

Figure 5. Bona Fide Tfh Cells Are Undetectable in Blood

Healthy individuals (n = 5) were vaccinated with seasonal influenza virus vaccines and blood was taken 7 days after vaccination. Tonsils were obtained from tonsillectomies. FACS plots show CXCR5, PD-1, and BCL6 expression in $CD4^+$ T cells (A and B) and CCR7 and PD-1 expression on cells gated from A (C) or BCL6 in cells gated from C (D). PBMCs and spleens from 8- to 12-week-old *sanroque* mice were analyzed. FACS plots show CXCR5, PD-1, and BCL6 expression in $CD4^+$ T cells (E and F) and CCR7 and PD-1 expression on cells gated from E (G) or BCL6 in cells gated from G (H). Statistics (n = 3–5) shows the results of individual human or mice with average values.

2009). Conversely, when naive OT-II cells with the *Roquin*$^{san/san}$ mutation were adoptively transferred, higher percentages of the $CCR7^{lo}PD\text{-}1^{hi}$ subset than those of WT OT-II cells were observed (Figure 4E). These observations indicate that the increase of the $CCR7^{lo}PD\text{-}1^{hi}$ subset in blood of *sanroque* mice reflects spontaneous and continuous Tfh differentiation. This observation further validates the usage of the $CCR7^{lo}PD\text{-}1^{hi}$ subset in blood as a marker for active Tfh differentiation in secondary lymphoid organs.

Bona Fide Tfh Cells Are Undetectable in Blood

The controversy of whether Tfh cells are present in blood (Craft, 2012) can be largely attributed to the ambiguous definition of Tfh cells (Yu and Vinuesa, 2010a). The most widely accepted phenotypic definition of Tfh cells is high expression of PD-1 and CXCR5 together with BCL6 (Yu and Vinuesa, 2010a). Blood from human subjects 1 week after vaccination contained $CD4^+$ T cells with detectable CXCR5 and PD-1 expression (Figure 5A). However, if amounts of CXCR5 and PD-1 expression on Tfh cells are defined by amount expressed on $CD4^+$ T cells in human tonsils, then very few, if any, Tfh cells could be detected in blood (Figure 5A). Similarly, T cells expressing BCL6 were absent in blood (Figure 5B). Furthermore, $CD4^+$ T cells with high amounts of CXCR5 and PD-1 expression were barely detectable in 26 patients with SLE and 38 patients with RA (data not shown). Even in *sanroque* mice, which present a massively expanded Tfh cell compartment in secondary lymphoid organ as a result of spontaneous Tfh differentiation (Vinuesa et al., 2005), Tfh cells in blood were again undetected (Figure 5E; Figure S5F).

In both human tonsils and mouse spleens with active Tfh programs, the $CCR7^{lo}PD\text{-}1^{hi}$ subset was readily detected in cells expressing intermediate amounts of CXCR5 (Figure 5C; Figure S5G). However, unlike their counterparts in blood, 2- to 3-fold increase of BCL6 expression was observed in $CCR7^{lo}PD\text{-}1^{hi}$ $CXCR5^+$ $CD4^+$ T cells in secondary lymphoid organs (Figure 5D; Figure S5H). Tfh cell differentiation is a multistage process (Crotty, 2011), and SAP-mediated stable T cell-B cell interaction strengthens ICOS signaling and BCL6 expression (Choi et al., 2011). We have previously shown that the differentiation of circulating $CXCR5^+$ $CD4^+$ T cells requires ICOS and BCL6, but not SAP (Figure 3). The expression of BCL6 in $CXCR5^+$ $CD4^+$ T cells in secondary lymphoid organs, but not in blood, further suggests that circulating $CXCR5^+$ $CD4^+$ T cells deviate from Tfh differentiation before stable T cell-B cell interaction for terminal Tfh differentiation.

Expansion of Circulating $CCR7^{lo}PD\text{-}1^{hi}$ $CXCR5^+$ $CD4^+$ T Cells in Patients with Autoimmune Diseases

The percentage of total $CXCR5^+$ Tfh-related cells in blood samples from healthy controls (HC) and patients with RA or SLE was similar and ranged from about 5% to 25% (Figure 6A; Figure S6B). In HC, the $CCR7^{lo}PD\text{-}1^{hi}$ subset among total $CXCR5^+$ $CD4^+$ T cells ranged from 9% to 21.5%, regardless of

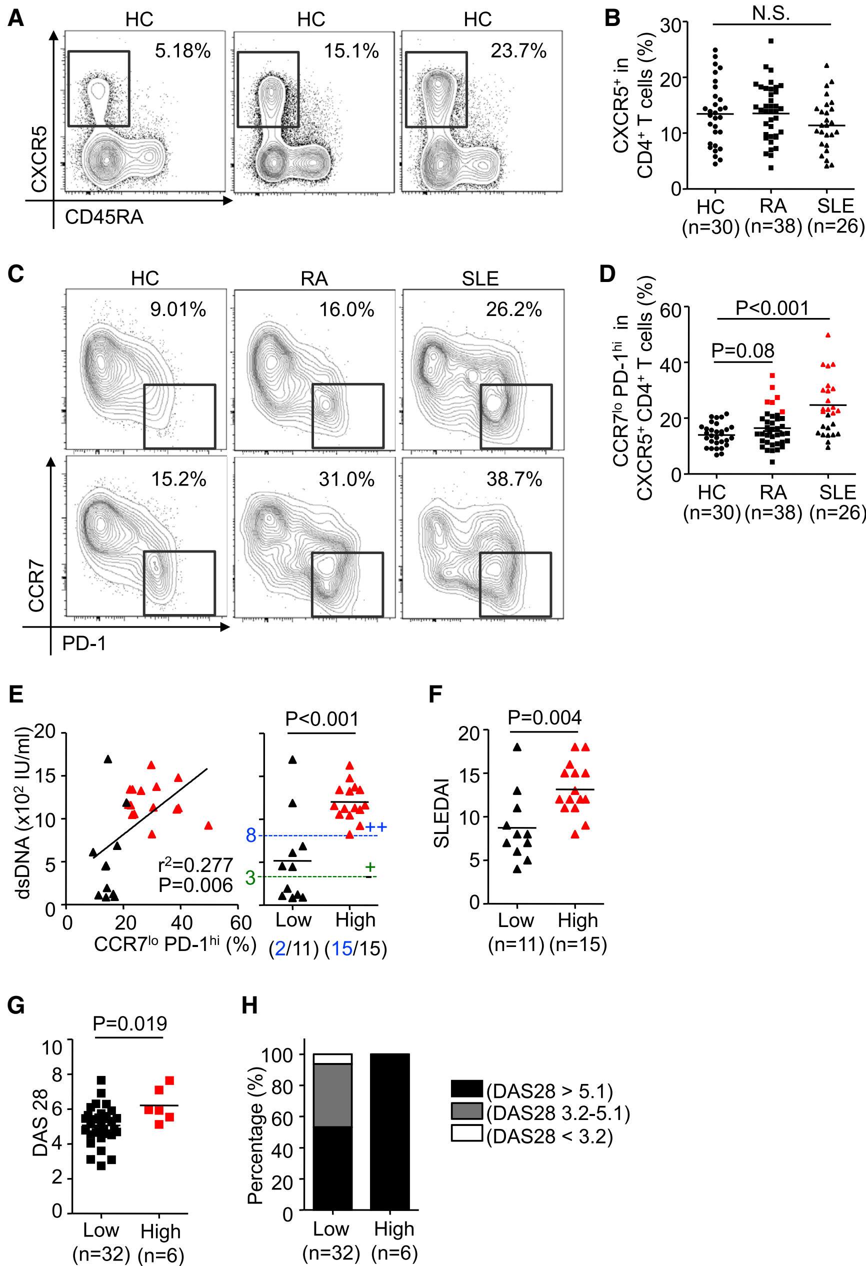

Figure 6. Increased Percentages of Circulating CCR7^{lo}PD-1^{hi} Cells in Autoimmune Patients with High Amounts of Autoantibodies and Severe Disease Activity

PBMCs were isolated from healthy controls (HC, n = 30), RA patients (n = 38), and SLE (n = 26) patients were analyzed.

(A and B) FACS plots and statistics show the percentages of CXCR5^{+} CD4^{+} T cells.

(C and D) FACS plots and statistics show the CCR7^{lo}PD-1^{hi} subset within CXCR5^{+}CD45RA^{-} CD4^{+} T cells from two representative individuals in indicated groups. Patients with CCR7^{lo}PD-1^{hi} percentages higher than HC are labeled in red.

(E) The correlation between the concentration of anti-dsDNA autoantibodies in serum and the percentages of CCR7^{lo}PD-1^{hi} cells was examined (left panel) and the classifications based on low or high CCR7^{lo}PD-1^{hi} percentages as defined in D (right panel). The thresholds for moderate and high titers are shown by green and blue lines, respectively. The numbers of patients with high titers are indicated in blue.

(F and G) The disease activity scores of individual SLE or RA patients in the CCR7^{lo}PD-1^{hi} low or high groups are shown.

(H) Distribution of low, medium, or high DAS28 RA patients within CCR7^{lo}PD-1^{hi} low or high groups. Statistics show the results of individuals with average values. See also Figure S5 and Table S1.

age (Figures S5A and S5B). Notably, several RA patients and more than half of SLE patients showed an increase in the percentage of CCR7^{lo}PD-1^{hi} cells to greater than 21.5% (labeled in red, Figure 6C; Figure S6D). The average percentage of CCR7^{lo}PD-1^{hi} subsets in SLE patients was significantly higher than those in HC (Figure 6D). The percentage of the CCR7^{lo}PD-1^{hi} subset cells within CXCR5^{+} CD4^{+} T cells did not correlate with either age or duration of disease in SLE patients (Figures S5C and S5D). However, SLE patients with a higher percentage of CCR7^{lo}PD-1^{hi} cells (>21.5%, high group) also presented with higher amounts of several key diagnostic autoantibodies for SLE, including autoantibodies against dsDNA (Figure 6E), Smith (Sm), and nucleosome (Figures S5E and S5F) than those with lower CCR7^{lo}PD-1^{hi} subset percentages (<21.5%, low group). Furthermore, all (15/15) SLE patients in the high group had high amounts of anti-dsDNA, whereas in the low group, only a small proportion (2/11) of patients did (Figure 6E). In addition, patients in this high group showed higher SLE disease-activity indexes (SLEDAI) (Bombardier et al., 1992) (Figure 6F). RA patients with high CCR7^{lo}PD-1^{hi} percentages also had higher disease activity scores (DAS28) (Prevoo et al., 1995) than those in the CCR7^{lo}PD-1^{hi} low group (Figure 6G) with all patients in the high CCR7^{lo}PD-1^{hi} group classified as high disease activity (Figure 6H). Whereas total CXCR5^{+} CD4^{+} T cells in blood are not indicative of disease activity, overrepresentation of the CCR7^{lo}PD-1^{hi} subset was associated with higher autoantibody titer and higher disease activity.

Circulating CCR7^{lo}PD-1^{hi} CXCR5^{+} Tfh Precursor Cells Promote Antibody Responses

The presence of CXCR5^{+} CD4^{+} T cells in blood suggests these cells are generated from draining secondary lymphoid organs and travel through nondraining secondary lymphoid organs. We then asked whether this could represent a mechanism to enhance Tfh differentiation and antibody response in nondraining secondary lymphoid organs.

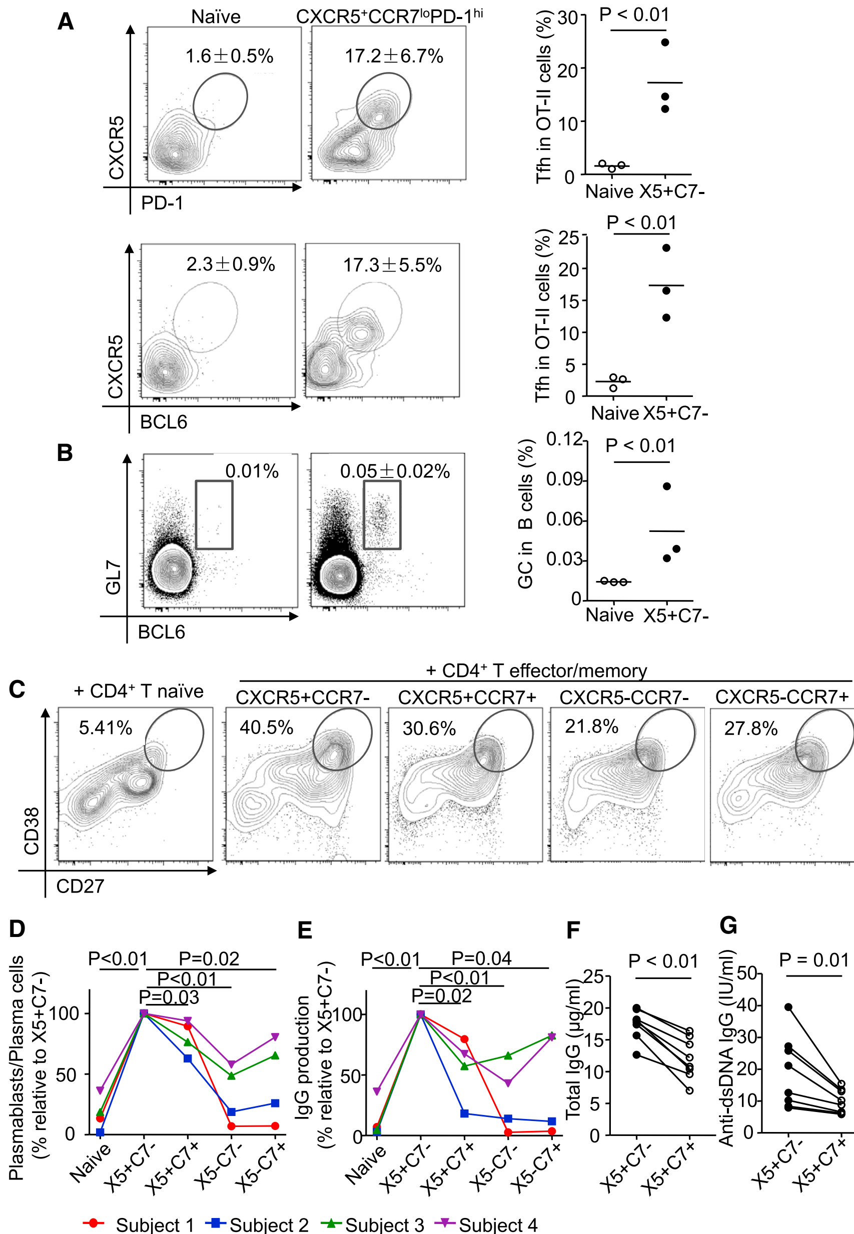

Figure 7. Circulating CCR7^{lo}PD-1^{hi} CXCR5^{+} Tfh Precursor Cells Promote Antibody Responses

(A and B) CD45.2 naive OT-II cells were i.v. injected into CD45.1 mice followed by i.p. immunization with OVA in alum. We sorted 60,000 CXCR5^{+} OT-II cells from blood of immunized mice at day 7 or 60,000 naive CD4^{+} T cells from unimmunized OT-II mice and adoptively transferred them into CD45.1 *Cd28*$^{-/-}$ mice, which were immunized i.p. with OVA in alum. Representative FACS plots and statistical analysis (n = 3) show Tfh differentiation and GC formation in spleens.

(C and D) We isolated 20,000–50,000 CD4^{+} T cells of indicated subsets from individuals 7 days after influenza vaccine and cocultured them with autologous memory B cells at 1:1 ratio for 4 days. Representative FACS plots and statistics (n = 4) showing the differentiation of plasmablasts or plasma cells. The production of total IgG in the coculture supernatants was shown in (E). The values displayed in (D) and (E) were normalized to the CXCR5^{+}CCR7^{-} group, which were set to 100%. We isolated 20,000–50,000 CD4^{+} T cell of indicated subsets from SLE patients and cocultured them with autologous memory B cells at 1:1 ratio for 4 days. Statistics (n = 8) showing the production of total IgG (F) and anti-dsDNA IgG (G). X5, CXCR5; C7, CCR7. See also Figure S6.

To test this, we transferred CD45.2 naive OT-II CD4^{+} T cells into CD45.1 congenic mice immunized s.c. with OVA in alum at the rear foot pad. This immunization led to Tfh differentiation and GC formation in the draining popliteal lymph node, but not the nondraining brachial lymph node. Six days after this immunization, a group of mice (R 1st + F 2nd) was immunized with OVA in alum at front foot pad as a boost. A control group (F 1st) received only transferred OT-II cells but without the priming immunization (Figures S6A and S6B). In the prime and boost group (R 1st + F 2nd), antigen-experienced OT-II cells in brachial lymph nodes rapidly differentiated into mature Tfh cells upon antigen reexposure to support fast GC formation 5 days after the boost immunization (Figures S6C and S6D). In contrast, despite stronger proliferation of naive OT-II cells in brachial lymph nodes of the control group (F 1st) than antigen-experienced OT-II cells in the prime and boost group (R 1st + F 2nd) (Figure S6B), the differentiation into mature Tfh cells was slower and there was no GC formation 5 days after the primary immunization (Figures S6C and S6D). This demonstrates an ongoing Tfh differentiation and GC response in the draining secondary lymphoid organ that could accelerate the Tfh differentiation and GC response in the nondraining secondary lymphoid organ once antigen is presented.

Circulating CCR7^{lo}PD-1^{hi} CXCR5^{+} Tfh precursor cells are generated early in the antibody response (Figure 1; Figures S4A and S4B). We next examined whether these cells are sufficient to accelerate GC formation. CD45.2 naive OT-II CD4^{+} T cells were transferred into CD45.1 congenic mice immunized i.p. with OVA in alum. Seven days after immunization, circulating CXCR5^{+} OT-II cells were predominantly CCR7^{lo}PD-1^{hi} (Figure 1F) and were isolated from blood and adoptively transferred into CD45.1 *Cd28*$^{-/-}$ mice that were immunized i.p. with OVA in alum. Five days after cell adoptive transfer, the CCR7^{lo}PD-1^{hi} CXCR5^{+} but not the control naive OT-II cells were able to differentiate into mature Tfh cells (Figure 7A) and initiate GC formation (Figure 7B), despite stronger proliferation of naive OT-II cells than the CCR7^{lo}PD-1^{hi} CXCR5^{+} OT-II cells (Figure S6E). This establishes that circulating CCR7^{lo}PD-1^{hi} CXCR5^{+} CD4^{+} T cells can rapidly differentiate into mature Tfh cells upon antigen reencounter and can accelerate GC formation.

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To further examine whether the potent helper function of CCR7^{lo}PD-1^{hi} CXCR5^{+} CD4^{+} T cells also exists in human, we sorted different subsets of circulating CD4^{+} T cells from the blood of healthy individuals 1 week after influenza vaccination. By coculturing with autologous memory B cells, CCR7^{lo}PD-1^{hi} CXCR5^{+} but not naive CD4^{+} T cells potently induced CD27^{hi}CD38^{hi} plasmablast or plasma cell differentiation and total immunoglobulin G (IgG) production. The helper function of CCR7^{lo}PD-1^{hi} CXCR5^{+} subset was best among all CD4^{+} T cell subsets examined (Figure 7C; Figure S7D). Similar results were also observed for the production of vaccine-specific IgG (Figure S6F). We next examined whether circulating CCR7^{lo}PD-1^{hi} CXCR5^{+} Tfh precursor cells might play a pathogenic role in supporting the production of autoantibodies in SLE patients. Due to lymphopenia in SLE patients, the number of cells that could be isolated was limited, and therefore we only compared two subsets: CCR7^{lo}PD-1^{hi} and CCR7^{hi}PD-1^{lo} CXCR5^{+} CD4^{+} T cells. When cocultured with autologous memory B cells, the CCR7^{lo}PD-1^{hi} subset was better than the CCR7^{hi}PD-1^{lo} subset in facilitating the production of both total IgG (Figure 7F) and IgG specific to dsDNA (Figure 7G). By accelerating GC formation in vivo and enhancing antibody production in vitro, circulating CCR7^{lo}PD-1^{hi} CXCR5^{+} Tfh precursor cells demonstrate their capability to promote antibody response.

DISCUSSION

There is great need for a reliable and fully validated method to identify an active Tfh program with blood, which is the most convenient and often the only available clinical sample. Monitoring active Tfh program will provide essential information to understand protective antibody responses during infection or vaccination and pathogenic antibody responses in autoimmune diseases. Here, the phenotypes and kinetics of Tfh cell-related populations in blood were characterized in detail in both animal models and human samples. Although bona fide Tfh cells were undetectable in blood, Tfh-related CXCR5^{+} CD4^{+} T cells were present in blood, ranging from 5% to 25% (Figure 6A). In both mice and humans, two major subsets were clearly identified within circulating CXCR5^{+} CD4^{+} T cells: the CCR7^{lo}PD-1^{hi} subset had a Tfh precursor phenotype, whereas the phenotype of the CCR7^{hi}PD-1^{lo} subset was characteristic of resting cells (Figure 2; Figure S2).

Stratification of circulating CXCR5^{+} CD4^{+} T cells reveals that it is the CCR7^{lo}PD-1^{hi} subset of circulating CXCR5^{+} CD4^{+} T cells that identifies active Tfh differentiation (Figure 4A). Indeed, a high percentage of CCR7^{lo}PD-1^{hi} cells were observed in Tfh-biased autoimmune *sanroque* mice (Figure 4C) and SLE patients with high autoantibody titers and severe disease activities (Figure 6). This new method represents a significant advancement in monitoring Tfh differentiation, when compared to previous approaches (Feng et al., 2011; Lindqvist et al., 2012; Simpson et al., 2010; Zhu et al., 2012). This new method also extends our knowledge on the heterogeneity of circulating CXCR5^{+} CD4^{+} T cells. In addition to the previous report that different subsets with Th1, Th2, or Th17 features possess discrete capabilities to support B cells to produce antibodies (Bentebibel et al., 2013; Morita et al., 2011), this current study demonstrates that the CCR7^{lo}PD-1^{hi} subset but not the CCR7^{hi}PD-1^{lo} subset correlates with active Tfh differentiation. Therefore, a two-dimensional approach to monitor circulating CXCR5^{+} CD4^{+} T cells might be required to understand both polarization and dynamics of Tfh differentiation in individuals.

The generation of both CXCR5^{+} CD4^{+} T cell subsets is dependent on ICOS and BCL6 but independent of SAP in both mouse and human (Figure 3), suggesting a model in which both subsets are primarily generated before GC formation. The ontogeny of circulating CXCR5^{+} CD4^{+} T cells rationalizes the logic that an increase of CCR7^{lo}PD-1^{hi} CXCR5^{+} Tfh precursor cells in blood represents an active Tfh differentiation in secondary lymphoid organs.

CD4^{+} memory T cells are capable of participating memory responses (Pepper and Jenkins, 2011). Tfh cells sorted from lymphoid organs were able to survive over a period of time and participate in the recall responses (Choi et al., 2013; Hale et al., 2013; Liu et al., 2012; Lüthje et al., 2012). Circulating CXCR5^{+} CD4^{+} helper T cells can enhance antibody responses in vitro (Breitfeld et al., 2000; Chevalier et al., 2011; Schaerli et al., 2000) and in vivo (Sage et al., 2013). Here, we showed that antigen-experienced blood CCR7^{lo}PD-1^{hi}CXCR5^{+} Tfh precursor cells circulated to nondraining secondary lymphoid organs and rapidly differentiated into mature Tfh cells to support fast GC formation upon antigen reencounter (Figure 7; Figures S6A–S6D). In addition, these Tfh precursor cells were better than other circulating CD4^{+} T cell subsets to provide B helper function in in vitro culture (Figure 7; Figure S6F). Tfh precursor cells are generated primarily in the early phase of the response but are not derived from Tfh cells in GCs. The ability of Tfh precursors to circulate to nondraining secondary lymphoid organs suggests a mechanism for the immune system to accelerate antibody responses in nondraining secondary lymphoid organs when infectious agents begin to spread systemically, in order to win the race with fast-replicating pathogens.

Using precursor CCR7^{lo}PD-1^{hi}CXCR5^{+} CD4^{+} T cells to identify active Tfh differentiation will meet a long-sought goal to monitor the activation status of an individual's Tfh program. The ability to monitor Tfh cells might lead to new strategies to harness an individual's Tfh cell program, paving the way to optimizing vaccination strategies and tailoring diagnosis and treatment of autoantibody-mediated diseases.

EXPERIMENTAL PROCEDURES

Mice

Bcl6$^{-/-}$, *Icos*$^{-/-}$, *Sh2d1a*$^{-/-}$, *Cd28*$^{-/-}$, OT-II, Sanroque (*Roquin*$^{san/san}$), BAFF-Tg (*Baff*$^{Tg/Tg}$), and CD45.1 mice were maintained on a C57BL/6 background and housed in specific pathogen-free conditions at Monash University or Garvan Institute animal facilities. All animal experiments were carried out under protocols approved by each institute's animal ethics committee.

Human Subjects

Detailed information of human samples is provided in Supplemental Information. Ethics approvals were obtained from each institute's human ethics committee.

PBMC Isolation, Cell Subset Purification, and Culture

Human PBMCs were collected into sodium heparin tubes (BD) and purified by Ficoll-Paque PLUS (GE healthcare) density gradient centrifugation. Mouse PBMCs were collected into sodium heparin saline buffer (Pfizer) and purified

by Lympholyte (Cedarlane Labs) density gradient centrifugation. Detailed information of cell subset purification and culture is provided in Supplemental Information.

Immunizations

To generate T-dependent antibody responses, we immunized mice i.p. with 2×10^9 SRBC (Veterinary Services, IMVS). For induction of T-dependent responses after adoptive transfer of OT-II cells, mice were i.v. injected with $3–10 \times 10^4$ $OVA^{323–339}$-specific OT-II $CD4^+$ T cells and immunized i.p. with 50 μg of OVA in alum or Sigma Adjuvant System or s.c. footpad with 25 ug of OVA in alum.

Flow Cytometry and ELISA

Detailed information is provided in Supplemental Information.

Statistical Analyses

All statistical analyses were carried out with Prism (version 5.0, GraphPad Software) or SPSS (version 16.0). The data are presented as mean ± SD or as percentages, where appropriate. For comparisons between two populations, unpaired two-tailed Student's t tests were performed. For comparisons between SAP-sufficient and SAP-deficient cells, paired two-tailed Student's t tests were performed. For correlation analyses, either Spearman's r or an r^2 value derived from Pearson's r was calculated and two-tailed p value was determined in the analysis of correlations. Clinical characteristics and certain laboratory values between patient groups were compared with chi-square analysis. p values less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.09.007.

AUTHOR CONTRIBUTIONS

J.H. and L.M.T. helped the experimental design, performed major experiments (J.H. for human experiments and L.M.T for human and mouse experiments), analyzed the data, and edited the manuscript. Y.A.L and X.H. helped with the experiments. C.S.M. performed experiments on XLP patient samples. K.V. and S.R. performed experiments on vaccinated subject samples. N.C., G.T.B., and A.K. performed preliminary experiments. Y.D., X.S., W.W., and C.W. helped to collect data on autoimmune patients. M.C.C. and D.S.R. helped to collect XLP patient samples. S.G.T., A.V., P.L.S., F.M., R.B., C.G.V., and C.R.M. provided important materials and reviewed the manuscript. Z.L. designed experiments and reviewed the manuscript. D.Y. designed experiments, performed experiments, analyzed the data, wrote the manuscript, and supervised the study.

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