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CXCR5 Expressing Human Central Memory CD4 T Cells and Their Relevance for Humoral Immune Responses

Nina Chevalier,*†‡ David Jarrossay,† Edwin Ho,* Danielle T. Avery,* Cindy S. Ma,* Di Yu,‡ Federica Sallusto,† Stuart G. Tangye,*‡ and Charles R. Mackay‡

High expression of CXCR5 is one of the defining hallmarks of T follicular helper cells (T<sub>FH</sub>), a CD4 Th cell subset that promotes germinal center reactions and the selection and affinity maturation of B cells. T<sub>FH</sub> is also expressed on 20–25% of peripheral blood human central memory CD4 T cells (T<sub>CM</sub>), although the definitive function of these cells is not fully understood. The constitutive expression of CXCR5 on T<sub>FH</sub> cells and a fraction of circulating T<sub>CM</sub> suggests that CXCR5<sup>+</sup> T<sub>CM</sub> may represent a specialized subset of memory-type T<sub>FH</sub> cells programmed for homing to follicles and providing B cell help. To verify this assumption, we analyzed this cell population and show its specialized function in supporting humoral immune responses. Compared with their CXCR5<sup>-</sup> T<sub>CM</sub> counterparts, CXCR5<sup>+</sup> T<sub>CM</sub> expressed high levels of the chemokine CXCL13 and efficiently induced plasma cell differentiation and Ig secretion. We found that the distinct B cell helper qualities of CXCR5<sup>+</sup> T<sub>CM</sub> were mainly due to high ICOS expression and pronounced responsiveness to ICOS ligand costimulation together with large IL-10 secretion. Furthermore, B cell helper attributes of CXCR5<sup>+</sup> T<sub>CM</sub> were almost exclusively acquired on cognate interaction with B cells, but not with dendritic cells. This implies that a preferential recruitment of circulating CXCR5<sup>+</sup> T<sub>CM</sub> to CXCL13-rich B cell follicles is required for the promotion of a quick and efficient protective secondary humoral immune response. Taken together, we propose that CXCR5<sup>+</sup> T<sub>CM</sub> represent a distinct memory cell subset specialized in supporting Ab-mediated immune responses.

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The ability of memory T cells to mediate a faster and more effective response to secondary pathogen challenge is a central feature of adaptive immunity (1). Memory CD4 T cells are heterogeneous with respect to their homing capacities and effector function (2–6). There are two major subsets, central memory T cells (T<sub>CM</sub>) and effector memory T cells (T<sub>EM</sub>), in both human and mice. T<sub>CM</sub> typically express CD62 ligand (CD62L) in secondary lymphoid organs, whereas T<sub>EM</sub> lack these lymphoid homing receptors but express "inflammatory" receptors such as CCR5 and CXCR3 to gain access to inflamed peripheral tissues (9). In terms of effector function, T<sub>EM</sub> are more differentiated and polarized than T<sub>CM</sub>, and provide an immediate immune protection by rapidly producing effector cytokines on antigenic stimulation. In contrast, T<sub>CM</sub> display hypoacetylated cytokine genes, higher proliferative potential, and greater resistance to apoptosis; they have a limited effector function and are rather destined for long-term protection (10–15). It is likely that T<sub>CM</sub> might generate new waves of effector cells in secondary immune responses, whereas T<sub>EM</sub> provide immediate protection (16, 17).

Within the subset of CCR7<sup>-</sup>CD45RA<sup>-</sup>CD4<sup>+</sup> T<sub>CM</sub> in human peripheral blood, ~20–25% expresses CXCR5 (18, 19). CXCR5 and its ligand CXCL13 are important for B cell follicle formation in secondary lymphoid organs (20). Furthermore, high expression of CXCR5 is one of the defining features of follicular B helper T cells (T<sub>FH</sub>). T<sub>FH</sub> cells are a specialized subset of CD4 T cells that localize to B cell follicles and germinal centers (GCs) in secondary lymphoid organs, where they promote the differentiation of B cells into memory B cells and plasma cells (PCs), and are crucial for the processes of somatic hypermutation of Ig variable-region gene segments and class-switch recombination (21–23). In both mice and human, T<sub>FH</sub> cells are clearly different from other Th lineages. They express high levels of IL-21, CXCL13, the transcription factor B cell lymphoma 6 (Bcl-6), and costimulatory molecules including programmed cell death 1 (PD1) and ICOS (21, 24–30).

The constitutive expression of CXCR5 on both T<sub>FH</sub> cells and a fraction of T<sub>CM</sub> suggests an association between CXCR5<sup>+</sup> T<sub>CM</sub> and the GC reaction. This is supported by the finding of a deficiency in both GCs in lymphoid tissues and circulating CXCR5<sup>+</sup> CD4<sup>+</sup> T cells in the blood of patients with mutations in CD40L or ICOS (31). However, a definitive role for these cells is still unresolved. Generally, CXCR5<sup>+</sup> T<sub>CM</sub> have been described as relatively unpolarized cells at an early stage in effector differentiation, lacking the expression of activation markers and costimulatory molecules (18, 19, 32, 33). Their uniform expression of CD27 is quite reminiscent of naive T cells (32), and except for IL-2, CXCR5<sup>+</sup> CD4 T cells are poorer cytokine producers compared with their CXCR5<sup>-</sup> T<sub>CM</sub> counterparts (18, 19, 32, 33).
Several studies have proposed that CXCR5+ TCM represent recently activated cells (18, 33, 34). This was mainly based on the observation that recall responses to tetanus toxoid were predominantly detected within the pool of CXCR5+ memory CD4 T cells isolated from volunteers vaccinated with tetanus 8–12 y ago, whereas 6 d after reimmunization, a marked proliferative response was especially observed in the CXCR5+ memory CD4 T cell pool (32, 34). Likewise, responses to allogeneic monocytes were more prominent for CXCR5+ compared with CXCR5- T cells before and after immunization with recall Ag (34). Moreover, CXCR5 expression is typically observed on all T cells after antigenic simulation both in vivo and in vitro (34–37). CXCR5 expression on T cells after stimulation is, however, only transient and irreversibly lost after their terminal differentiation into different effector Th lineages like Th1, Th2, Th17, or Th22. An over, CXCR5 expression is typically observed on all T cells after immunization with recall Ag (34). More-

quick and efficient protective humoral immune response.

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peripheral blood CXCR5+ CD4 T cell subsets (29), suggesting that CXCR5+ TCM follow a differentiation pathway distinct from that of Tfh cells. Despite this, the observation of a parallel reduction in GCs and circulating CXCR5+ CD4 T cells in ICOS and CD40L-deficient patients (31) suggests there might exist an association between the GC reaction and peripheral blood CXCR5+ CD4 T cells. To obtain insight into the function of human CXCR5+ TCM in peripheral blood, we performed a detailed characterization of this cell population as compared with their peripheral blood CXCR5+ counterpart, as well as bona fide Tfh cells present in human tonsils. We used different stimulatory and costimulatory conditions to carefully study the differentiation of these cell subsets and set the focus on clarifying the relation between CXCR5+ TCM, Tfh, and the humoral immune response. In this study, we show that CXCR5+ TCM represent a distinct memory cell population specialized in supporting B cell-related immune responses. Compared with their CXCR5- TCM counterparts, CXCR5+ TCM efficiently induced PC differentiation and Ig secretion, and displayed a different profile in terms of cytokine and costimulatory molecule expression. The observation that the adoption of B cell helper attributes by CXCR5+ TCM required cognate interactions with B cells suggests that a preferential recruitment of these cells represents a recent recruitment of these cells may be correct for some,

but not all, of these cells. The expression of CXCR5 on a subset of peripheral blood TCM as well as tonsillar Tfh cells, begs the question of whether circulating CXCR5+ CD4 T cells represent recently activated cells may be correct for some,

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were examined for surface phenotype and intracellular cytokine expression as described earlier, and for secretion of IgM, IgG, and IgA by ELISA (30, 41, 42). Activation of CD4 T cells by different APCs was performed by incubating purified TSST-responsive Vβ2+ CD4 T cells for 24 h with autologous cDCs, pDCs, and B cells. For this, sorted cDCs, pDCs, and B cells were first matured for 14 h in complete medium supplemented with LPS (100 ng/ml, from *Escherichia coli*; Sigma), CpG 2006 DNA (5 μg/ml; Molecular Probes), or for (FcγRII)2 goat anti-human IgM/IgG/IgA (H+L) (Jackson Immunoresearch, PA) and ionomycin (Sigma) for 1 h in the presence of 100 ng/ml TSST-1 (Toxin Technology) before purified CD4 T cell subsets were added. Purified B cells were cultured with recombinant CD40L (41, 42) alone or together with 100 U/ml human IL-10 (DNAX), 50 ng/ml human IL-21 (PeproTech), 50 ng/ml human IL-17A, or 100 U/ml human IFN-γ (PeproTech), and levels of secreted Ig were measured by ELISA as described earlier.

ELISA, FACS analysis, and intracellular cytokine staining

Cytokine concentrations in culture supernatants were assessed by ELISA according to standard protocols and analyzed with the Softmax program. Total Ig produced in culture supernatants were measured using IgG-, IgA-, and IgM-specific ELISA; Abs were used from Southern Biotech. Certified Reference Material 470 (ERM-DA470, Institute for Reference Materials and Measurements) (43) and human Ig standards (Sigma) were used as standard material for Ig isotype quantification. Intracellular cytokine expression was detected after restimulation of cells with PMA and ionomycin in the presence of 10 ng/ml brefeldin A (Sigma-Aldrich). Cells were then fixed and permeabilized with BD Cytofix/Cytoperm Plus (BD Bioscience) according to the manufacturer’s instructions and incubated with the respective Abs. Abs from CD40L expression, requiring 1-h stimulation in the presence of PMA and ionomycin, the expression of chemokine receptors and surface and costimulatory molecules was performed without further restimulation. After staining, cells were washed, acquired on a FACSCalibur (BD Biosciences) or FACScantoII (BD Biosciences), and analyzed using the FlowJo software (Tree Star).

Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Random hexamer primers and an Moloney murine leukemia virus reverse transcriptase kit (Invitrogen) were used for cDNA synthesis. Transcripts were quantified by real-time quantitative PCR on an ABI PRISM 7700 Sequence Detector with predesigned TaqMan Gene Expression Assays and reagents according to manufacturer’s instructions (Perkin-Elmer Applied Biosystems). Probes with the following Applied Biosystems assay identification numbers were used: GAAT-3 Hs00231122_m1; II-1F Hs00174122_m1; ROCHE Hs01076112_m1; TBX21 Hs00230436_m1; Bcl-6 Hs00277037_m1; II-21 Hs00222327_m1; II-10 Hs00961622_m1; cMAF Hs00193510_m1; cXCL1 Hs00757930. For each sample, mRNA abundance was normalized to the amount of 18S rRNA and is presented in arbitrary units. Alternatively, RT-PCR primers (Integrated DNA Technologies, Coralville, IN) were designed using the Roche UPL Primer Design software (Roche Dye Terminator cycle sequencing kit on an ABI PRISM 7700 Sequence Detector with predesigned TaqMan Gene Expression Assays and reagents according to manufacturer’s instructions). Real-time quantitative PCR was carried out using the Roche LightCycler 480 Probe Master Mix and the Roche LightCycler 480 System. All reactions were standardized to the level of expression of the housekeeping gene GAPDH.

Microarray analysis

Naïve CD4 T cells, T EM, and CXCR5+ and CXCR5− T CM were purified as described earlier and total RNA extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. For each population, biotin-labeled cRNA was amplified from 30 ng RNA using the two-cycle cDNA synthesis kit (Affymetrix) as per manufacturer’s instructions. Biotin-labeled cRNA was hybridized to U133plus2 Affymetrix arrays, which were then washed, stained, and scanned as per the manufacturer’s protocol. The raw Affymetrix array data were normalized by the RMA method using GeneSpring GX 7.3.1 software (Agilent). Detailed descriptions of each microarray experiment are provided online at http://www.ncbi.nlm.nih.gov/geo (accession number GSE26928).

Statistical analysis

Differences between data sets were analyzed as indicated by either Student’s *t* test or one-sample *t* test with a hypothetical mean value of 1.

Results

**CXCR5+ T CM are more efficient at providing B cell help than are CXCR5− T CM**

It has been well established that CXCR5 expression is associated with an increased capability of human tonsillar CD4+ T cells to provide B cell help and support Ig production (Supplemental Fig. 2) (29, 30, 33, 41). We therefore asked whether CXCR5+ T CM, as compared with CXCR5− T CM, were more efficient in inducing PC differentiation and Ig secretion when cocultured with B cells. To this end, sorted TSST-responsive Vβ2+CXCR5+ or Vβ2+CXCR5− T CM were cocultured with autologous B cells in the presence of TSST for 5–6 d. Both T cell subsets induced a CD38+CD20+ plasmablast phenotype from cocultured B cells (Fig. 1A, 1B) and supported Ig secretion (Fig. 1C, 1D). However, PC differentiation and Ig secretion were significantly increased when B cells were cultured with CXCR5+ T CM compared with CXCR5− T CM. Comparable results were also achieved when CXCR5+ and CXCR5− T CM were cocultured in the presence of naive allogeneic B cells and anti-CD3 and anti-CD28 mAbs (Supplemental Fig. 2) or in the presence of allogeneic B cells without further stimulation (as described later). Considering the absolute number of B cells recovered after culture on CXCR5+ or CXCR5− T CM, we observed no difference between the two subsets in our allogeneic coculture (Supplemental Fig. 3) and only minor differences in the autologous coculture experiments (data not shown). We therefore assume that increased induction of plasmablast differentiation underlies the increased Ig secretion in the presence of CXCR5+ T CM, which is consistent with the generation of a greater frequency of cells with a CD38+CD20+ phenotype by CXCR5+ T CM compared with CXCR5− T CM (Fig. 1A, 1B).

We further used the earlier described allogeneic coculture approach to compare B cell helper capabilities of peripheral blood CXCR5+ and CXCR5− T CM directly with tonsil CD4 T cell subpopulations (naive CD4+CD45RA−CXCR5−, CD4+CD45RA−CXCR5+, CD4+CD45RA−CXCR5+ and CD4+CD45RA−CXCR5− T cells). When cultured in the presence of splenic naïve B cells and anti-CD3/CD28 mAbs, tonsillar CXCR5+ and CD4 T cells yielded the highest amounts of Ig secretion among tonsillar CD4 T cell subsets (Supplemental Fig. 2), consistent with previous studies reported by Ma et al. (30) and Rasheed et al. (29). The reduced “help” provided by T FH cells most probably reflects their limited proliferative capacity and increased susceptibility to apoptosis because of elevated expression of CD95, PD1, and other regulatory molecules such as BTLA and CD200 (all of which are absent from T CM cells) (29, 30, 44). Interestingly, peripheral blood cells were more efficient in inducing Ig secretion than all tonsillar T CM cell subsets (Supplemental Fig. 2). However, these results need to be interpreted with caution. Ideally, tonsillar and peripheral blood cell subsets from the same donor should be compared; however, it is logically challenging to obtain blood and tissues simultaneously from the same individual. Furthermore, because they originate from highly inflamed tissue, tonsillar CD4 T cells may mostly be at a state of final differentiation, prone to apoptosis and with less proliferative capacity than T CM.

**CXCR5+ and CXCR5− T CM are differently polarized to distinct effector lineages**

We next wondered whether CXCR5 expression on circulating CD4 T cells correlates with higher expression of molecules that are important for B cell–related immune responses and are typically upregulated on T FH cells (18, 19, 21, 24, 29, 30, 35). In previous studies, the association between CXCR5 expression on circulating CD4 T cells and Th-lineage polarization mainly focused on Th1 and Th2 cells (18, 32, 33). Recently, however, a growing number of additional Th lineages, T FH, Th9, Th22, and Th17, have been described. Thus, our first aim was to identify whether, within the central memory population, CXCR5+ T CM are skewed toward the T FH effector lineage rather than any other Th lineage.

We assessed global gene expression patterns in naïve CD4 T cells, CXCR5− T CM, and CXCR5+ T CM, and T EM using microarray...
analyses (Fig. 2). As expected, naive CD4 T cells displayed a less and T EM a more polarized phenotype than T CM. These differences were most clear with respect to transcription factors (Bcl-6, cMAF, GATA-3, RORγt, RORγt, signaling lymphocyte-activation molecule-associated protein [SAP], T-bet), chemokine and cytokine receptors (CCR5, CCR6, CCR7, CCR10, CCR4, CRTC2, IL-23R), and costimulatory molecules (CD40L, CD57, CTLA-4, Fas, ICOS, OX40, PD1), whereas transcript levels of cytokines (CXCL13, IFN-γ, IL-2, IL-4, IL-10, IL-17a, IL-21, IL-22) were generally low. The gene expression profiles of CXCR5 + and CXCR5 + TCM showed only minor differences, with CXCR5 + TCM appearing to be more differentiated than CXCR5 − TCM. Key TFH markers such as signaling lymphocyte-activation molecule-associated protein (SAP), Bcl-6, and CXCL13 were, however, hardly expressed by any of the subsets examined, consistent with the data reported by Rasheed et al. (29).

To further confirm these observations, we examined highly purified CXCR5 + versus CXCR5 + TCM for differential expression of Th-lineage–specific cytokines, surface markers/costimulatory molecules, and transcription factors by quantitative RT-PCR, flow cytometry, and ELISA. We used highly purified tonsillar CD4 + CD45RA − CXCR5hi TFH cells as a control, which typically express highest levels of B cell helper-associated molecules (IL-10, IL-21, OX40, PD1, ICOS, Bcl-6, and CXCL13) (Supplemental Figs. 4–6) (18, 19, 21, 24, 29, 35).

Cytokine expression was assessed by flow cytometry after polyclonal stimulation with PMA and ionomycin for 5 h (Fig. 3A, Supplemental Fig. 6, Table I), whereas cytokine secretion was measured by ELISA after 24-h stimulation with plate-bound anti-CD3 and PdBu (Fig. 3B, Supplemental Fig. 7). For IL-4, IL-10, and IL-21, we additionally compared mRNA levels in CXCR5 + and CXCR5 + TCM after 3- to 4-h stimulation with PMA and ionomycin (Fig. 3C), because IL-10 and IL-4 were hardly detectable by flow cytometry or ELISA, respectively, and IL-21 was not reliably detectable by ELISA as it was probably consumed by the cells themselves (data not shown). Within the two subsets, CXCR5 + TCM generally displayed a higher cytokine-producing capacity compared with CXCR5 + TCM, IL-17, IL-22, IL-4, and IFN-γ were all produced at higher levels by CXCR5 + TCM, whereas IL-21 was equally expressed between the two subsets. IL-10 was the only cytokine found to be increased in CXCR5 + TCM.

Previous studies revealed preferential expression of CCR3 and CCR4, chemokine receptors associated with Th1 and Th2 cells, respectively (45, 46), on CXCR5 − TCM (32). We therefore analyzed expression of a panel of chemokine receptors and surface molecules on highly purified CXCR5 + TCM and CXCR5 + TCM (Fig. 3D). CRTi2 and CCR10, which are typically expressed on IL-22–secreting cells (47), were increased on CXCR5 + TCM.

FIGURE 2. Differentially expressed genes in peripheral blood subsets of CD4 T cells. Heat map showing relative expression levels of selected genes related to cell migration, signaling, transcription, and differentiation. Data are derived from two independent donors for each T cell subset examined on separate chip analyses (marked as 1 and 2).
CCR6 and IL-23R, preferentially expressed by Th17 cells (48–50), were equally expressed between the two subsets. No differential expression was furthermore observed for CD57, ICOS, and PD1 (Table II, Supplemental Fig. 5) and the costimulatory molecules CD40L and OX40 (Table II, Supplemental Fig. 5).

Consistent with a more pronounced resemblance to Th1, Th2, Th22, and Th17 features, CXCR5⁺ TCM displayed higher levels of transcription factors GATA-3 and RORγt, whereas T-bet was equally expressed between the two subsets (Figs. 2, 3, Supplemental Fig. 4). No difference in expression of the Tfh-associated markers CXCL13 and Bcl-6 was observed between CXCR5⁺ and CXCR5⁻ TCM, with CXCL13 being below detection level in most cells (Fig. 3C, Supplemental Fig. 4). The only transcription factor that was increased in CXCR5⁺ over CXCR5⁻ TCM was cMAF (Figs. 2, 3C). In summary, CXCR5⁺ TCM display a relatively unpolarized phenotype and lack expression of key Tfh markers when examined ex vivo.

CXCR5⁺ TCM lose their unpolarized phenotype after in vitro stimulation and display a cytokine expression profile distinct from CXCR5⁻ TCM cells

We hypothesized that the true relevance of CXCR5⁺ TCM cells might be evident only after activation because cells may differentiate and adopt effector function. Previous studies by Rivino et al. (32) established that chemokine receptors on TCM could discriminate cells with predetermined fates. CXCR3 and CCR4 were expressed on two subsets of TCM cells, which spontaneously differentiated to Th1 and Th2 effector cells, respectively (32). We therefore asked whether expression of CXCR5 on TCM identifies them as pre-Tfh memory cells that are capable of rapidly adopting Tfh-related features and functions when engaged in immune responses.

Based on the rapid kinetics of the memory response on secondary Ag encounter, we assessed cytokine production by purified CXCR5⁺ and CXCR5⁻ TCM after in vitro stimulation for 48 h. Considering the importance of ICOS costimulation in GC reactions and secondary humoral immune responses (51–53), we stimulated the two cell subsets on CD3/CD28 and CD3/ICOS-L, and measured production of IL-17, IL-10, IL-22, IL-21, IL-4, and IFN-γ by ELISA (Fig. 4A) and/or intracellular cytokine staining (Fig. 4B–E) and/or RT-PCR (Supplemental Fig. 8). Because of weak intracellular expression of IL-22 and IL-4 after 48 h of stimulation, the cells were additionally examined after a consecutive stimulation on plate-bound CD3/CD28 or CD3/ICOS-L for 2 d, followed by 3-d expansion in the absence of stimulus.

Under in vitro stimulatory conditions, CXCR5⁺ TCM no longer presented themselves as unpolarized or resting cells, but rather displayed a profile and stimulus responsiveness that was distinct from CXCR5⁻ TCM. Most prominent was the increased expression of both IL-10 and IL-17 by CXCR5⁺ TCM especially after stimulation on CD3/ICOS-L. CXCR5⁺ TCM furthermore expressed more IL-21 compared with CXCR5⁻ TCM, highest levels were, however, achieved in the presence of CD3/CD28. In accordance with their polarization when examined ex vivo, CXCR5⁺ TCM also expressed higher IL-22 levels after stimulation. The same trend was detected for IL-4, albeit its production by both TCM subsets was very low. The results for IFN-γ expression, and to a lesser extent for IL-17 expression, as measured by flow cytometry and secretion as measured by ELISA were not fully concordant. We believe this can be explained by the different techniques used to determine IL-17 and IFN-γ production. Whereas ELISA measures the accumulative amount of cytokines produced during a distinct period, intracellular cytokine staining rather reveals the potential of activated cells to express cytokines after 5-h restimulation with PMA/ionomycin. Even if cytokine expression levels and the amount of cytokine secreted in culture supernatants are not fully concordant, the results clearly show that IFN-γ was expressed and secreted at high levels by both subsets, with CXCR5⁺ TCM again revealing high responsiveness toward ICOS-L stimulation yielding the greatest expression of IFN-γ.

Expression of CXCL13, ICOS, and cMAF are preferentially increased in CXCR5⁺ TCM after in vitro stimulation

Because of the higher capacity of CXCR5⁺ TCM to produce B helper cytokines, especially IL-10, and to a lesser extent IL-21, we next asked whether in vitro stimulation also resulted in a preferential upregulation of expression of molecules that are typically associated with B cell help and expressed by Tfh cells, such as Bcl-6, CXCL13, CD57, ICOS, CD40L, or OX40 (Supplemental Figs. 4–6) (21–23, 30).

The transcription factor Bcl-6 is highly expressed by Tfh cells (Supplemental Fig. 4) (24, 29) (30) and is required for the Tfh-lineage differentiation (54–56). In vitro stimulation with CD3/CD28 or CD3/ICOS-L resulted in a Bcl-6 upregulation by both CXCR5⁺ and CXCR5⁻ TCM with only a very slight trend for greater expression by CXCR5⁺ TCM (Fig. 5A).

CXCL13 is another molecule that is produced in high quantities by Tfh cells (Supplemental Fig. 4) (29) and is abundantly expressed in the GC light zone (57). When examined ex vivo, CXCL13 expression was detectable neither in CXCR5⁺ nor CXCR5⁻ TCM. However, in vitro stimulation with CD3/ICOS-L resulted in a marked increase of CXCL13 mRNA levels particularly in CXCR5⁺ TCM compared with CXCR5⁻ TCM (Fig. 5B). This heightened responsiveness of CXCR5⁺ TCM to ICOS-L costimulation for production of CXCL13 was also confirmed by ELISA (Fig. 5C).

Tfh cells typically express high levels of ICOS (Supplemental Fig. 5) and CD40L (30), costimulatory molecules involved in interactions between T and B cells. More than 85–95% of both CD4 T cell subsets expressed CD40L or ICOS at 48 h post-stimulation (Fig. 5D). Generally, CD3/CD28 stimulation resulted in a stronger and quicker ICOS upregulation than CD3/ICOS-L stimulation (data not shown), which might be explained by a possible receptor downregulation caused by engagement. Although CXCR5⁺ and CXCR5⁻ TCM did not differ with respect to CD40L expression levels, CXCR5⁺ TCM displayed a greater percentage of ICOS⁺ cells (Fig. 5D), which might explain the observed higher responsiveness toward ICOS-L stimulation (Figs. 4A–E, 5B, 5C).

We further examined expression of CD70, which in cooperation with IL-10 directs the differentiation of CD27⁺ memory B cells toward PC (58), and of CD57, which is highly expressed by the subset of human Tfh cells (19, 24, 25, 29, 30). CD70 displayed preferentially in the presence of ICOS-L (Fig. 5D). However, in vitro stimulation with CD3/ICOS-L resulted in a marked increase of CXCL13 mRNA levels particularly in CXCR5⁺ TCM compared with CXCR5⁻ TCM (Fig. 5B). This heightened responsiveness of CXCR5⁺ TCM to ICOS-L costimulation for production of CXCL13 was also confirmed by ELISA (Fig. 5C).

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Because the high IL-17 secretion by CXCR5⁺ TCM was quite surprising, we questioned whether this was paralleled by an increased upregulation of the Th17-lineage–specific transcription factor RORγt or cMAF. The latter was recently shown to be induced by ICOS engagement and to regulate proliferation of and IL-21 production by Th17 and Tfh cells (59). It was also expressed at slightly higher levels in CXCR5⁺ TCM ex vivo (Fig. 3C). Whereas after 48 h of in vitro stimulation RORγt mRNA levels were nearly equivalent between the subsets, cMAF mRNA levels again reached higher expression levels in CXCR5⁺ TCM and preferentially in the presence of ICOS-L (Fig. 5E).
CXCR5^+ and CXCR5^- T_CM are differently polarized; a T_{FH}-like polarization is, however, apparent in neither subset. A, Freshly purified CXCR5^+ and CXCR5^- T_CM were stimulated by PMA and ionomycin for 5 h, and expression of IL-21, IL-4, IFN-γ, IL-17, and IL-22 analyzed by flow cytometry. Cytokine expression levels are shown as fold difference of CXCR5^- T_CM as compared with CXCR5^+ T_CM. B, Freshly purified CXCR5^+ and CXCR5^- T_CM were stimulated with anti-CD3 and PdBu, and IL-10, IFN-γ, IL-17, and IL-22 measured in 24-h culture supernatant. Cytokine levels are shown as fold difference of CXCR5^- T_CM as compared with CXCR5^+ T_CM. C, The abundance of RNA encoding for IL-17, IL-4, GATA-3, RORγt, T-bet, Bcl-6, IL-21, IL-10, and cMAF was determined by quantitative PCR after purification of CXCR5^+ and CXCR5^- T_CM and 4-h stimulation with PMA and ionomycin. 

**FIGURE 3.** CXCR5^+ and CXCR5^- T_CM are differently polarized; a T_{FH}-like polarization is, however, apparent in neither subset. A, Freshly purified CXCR5^+ and CXCR5^- T_CM were stimulated by PMA and ionomycin for 5 h, and expression of IL-21, IL-4, IFN-γ, IL-17, and IL-22 analyzed by flow cytometry. Cytokine expression levels are shown as fold difference of CXCR5^- T_CM as compared with CXCR5^+ T_CM. B, Freshly purified CXCR5^+ and CXCR5^- T_CM were stimulated with anti-CD3 and PdBu, and IL-10, IFN-γ, IL-17, and IL-22 measured in 24-h culture supernatant. Cytokine levels are shown as fold difference of CXCR5^- T_CM as compared with CXCR5^+ T_CM. C, The abundance of RNA encoding for IL-17, IL-4, GATA-3, RORγt, T-bet, Bcl-6, IL-21, IL-10, and cMAF was determined by quantitative PCR after purification of CXCR5^+ and CXCR5^- T_CM and 4-h stimulation with PMA and ionomycin. mRNA expression levels are shown as fold difference of CXCR5^+ T_CM as compared with CXCR5^- T_CM. A–C, Each symbol represents cells obtained from different donors. The p value was calculated using one-sample t test of the fold difference between CXCR5^+ and CXCR5^- T_CM with a hypothetical mean value of 1 (CXCR5^+ T_CM = CXCR5^- T_CM). D, Freshly purified CXCR5^+ and CXCR5^- T_CM were analyzed by flow cytometry for the expression of surface makers as indicated. Each symbol represents cells obtained from different donors. The p value was calculated using paired Student t test.

Increased levels of ICOS expression and IL-10 secretion by CXCR5^+ T_CM account for their greater B cell helper function

So far, we observed greater B cell helper function by CXCR5^+ T_CM as compared with their CXCR5^- counterpart. Even if CXCR5^- T_CM did not differentiate into archetypal T_{FH} cells under the chosen conditions, they at least adopted a phenotype that was different from CXCR5^- T_CM and may point toward their specialized commitment to support humoral immune responses. We next wondered whether stimulation in the presence of B cells would result in the acquisition of the same phenotypic characteristics and hence explain the better B cell helper functions of CXCR5^+ T_CM.

After 5 d of stimulation on B cells, CXCR5^+ T_CM again expressed significantly increased levels of IL-10 as compared with CXCR5^- T_CM (Fig. 6A). IL-21 and IFN-γ levels were expressed at similar levels by both CXCR5^+ and CXCR5^- T_CM, whereas plate-bound CD3/CD28/ICOS-L stimulation resulted in a slightly higher expression of IL-21 by CXCR5^+ T_CM; IL-17 and IL-4 were only weakly expressed, but in accordance with stimulation on plate-bound CD3/CD28 and CD3/ICOS-L, CXCR5^+ T_CM displayed slightly decreased IL-4 but increased IL-17 levels (Fig. 6A). As already observed after plate-bound stimulation, CXCL13 was significantly upregulated in CXCR5^+ T_CM relative to CXCR5^- T_CM (Fig. 6B). Activated CXCR5^+ T_CM also generated a greater frequency of cells exhibiting an ICOS^hiCD57^- phenotype than CXCR5^- T_CM (Fig. 6C). Bcl-6 was not differentially regulated between the two subsets (Fig. 6B). After 5 d of coculture with B cells, we furthermore found a significant downregulation of CD40L and CD70 on both CXCR5^+ and CXCR5^- T_CM, most probably caused by receptor-mediated downregulation (data not shown) (60).

In view of the fact that different stimulatory conditions may result in a differential expression of genes, cytokines, and co-stimulatory molecules, and considering the results of 48 h on plate-bound CD3/CD28/ICOS-L stimulation and 5-d stimulation in the presence of B cells side by side, we conclude that on stimulation, CXCR5^+ and CXCR5^- T_CM mainly differ in their responsiveness toward ICOS-L stimulation, production of IL-10, IL-17, and CXCL13, and the adoption of an ICOS^hi phenotype. CD40L, Bcl-6, IL-21, and IFN-γ were more or less equally expressed by both subsets, consistent with the fact that CXCR5^- T_CM were also able to induce considerable PC differentiation and Ig secretion by cocultured B cells.

To clarify the effects of IL-21, IL-10, IFN-γ, and IL-17 on Ig secretion by human B cells, naive and memory B cells were stimulated with recombinant CD40L alone or together with IL-10, IL-17, IL-21, or IFN-γ, and secretion of IgM, IgG, and IgA determined after 12 d. This clearly showed that IL-21 was the most efficient inducer of Ig secretion by CD40L-stimulated B cells. Modest Ig secretion could also be achieved in the presence of IL-10; however, neither IL-17 nor IFN-γ had any effect on Ig secretion above that observed for CD40L alone (Supplemental Fig. 9).

Based on these results, we conclude that IL-21 secretion by both T_CM subsets significantly contributes to the induction of Ig secretion and PC differentiation. Because of comparable levels of Bcl-6, CD40L, and IL-21 in CXCR5^+ and CXCR5^- T_CM, we propose that in CXCR5^+ T_CM, the higher expression of ICOS and production of the B helper cytokine IL-10 may underlie their increased capacity to provide B cell help. To test this possibility, we cultured CXCR5^+ T_CM with allogeneic B cells in the presence of neutralizing anti–IL-10 and anti–ICOS-L mAbs. Blockade of ICOS–ICOS-L interactions resulted in a marked decrease of both PC differentiation and Ig secretion as compared with B cell–T cell cocultures without addition of neutralizing mAbs (Fig. 6D, 6E). Neutralization of IL-10 also reduced CXCR5^+ T_CM–induced B cell...
differentiation in some experiments (Fig. 6D, 6E). The variable effects of the anti–IL-10 mAbs probably reflected the donor-to-donor variation in IL-10 production, as shown in Fig. 6A (i.e., 4–32% IL-10+ cells). Moreover, variability of Ig secretion both in the absence and presence of neutralizing mAbs is not surprising because donor variability is common and to be expected when working with samples from an outbred human population; likewise, different experimental approaches or culture conditions may result in variable results.

Our data clearly show an association between CXCR5+ TCM and B cell helper function. We therefore hypothesized that, as compared with CXCR5+ TCM, CXCR5+ TCM might be preferentially recruited to CXCL13-rich B cell follicles, and this increases their interaction with B cells rather than other APCs such as dendritic cells (DCs), primarily located in the T cell zone. A faster and more numerous interaction between

**FIGURE 4.** In vitro stimulation of CXCR5+ and CXCR5− TCM induces different cytokine expression profiles, with CXCR5+ TCM displaying a notable responsiveness toward ICOS-L costimulation. A and B, Cytokine production by freshly purified CXCR5+ and CXCR5− TCM stimulated in vitro for 48 h with either anti-CD3/CD28- or anti-CD3/ICOS-L-coated plates, followed by cytokine ELISA of culture supernatants (A) and intracellular cytokine staining after restimulation with PMA and ionomycin (B). Each symbol represents cells obtained from different donors; p value was calculated using paired t test; small horizontal bars indicate the mean. C–E, Representative dot blots of cytokine production by freshly purified CXCR5− and CXCR5+ TCM treated as described for B.

**CXCR5+ TCM require cognate interaction with B cells to become highly efficient B cell helpers**
CXCR5+ TCM and B cells might then result in sufficient and more rapid Ig secretion in secondary immune responses. Accordingly, the association between CXCR5 expression and an increased migration toward CXCL13 has been shown for both tonsilar and peripheral blood CD4 T cells (18, 19, 31, 34). We asked whether stimulation of CXCR5+ TCM by B cells is as efficient as stimulation by DCs to induce B cell helper capabilities and the observed high expression of CXCL13. To this end, we stimulated purified TSST-responsive Vβ2+ CXCR5+ and CXCR5+ TCM in the presence of TSST on either autologous peripheral blood B cells, cDCs, or pDCs that had been matured overnight by incubation with F(ab)2; anti-human IgM/IgA/IgG, LPS, or CpG, respectively. After 24 h of stimulation, CD4+ T cells were resorted and either subjected to quantitative PCR analysis for differential CXCL13 mRNA expression (Fig. 7A) or recultured with allogeneic B cells for another 5 d to determine the capacity of differently stimulated T cells to induce PC differentiation and Ig secretion (Fig. 7B, 7C). Consistent with the results obtained after stimulation of CXCR5+ and CXCR5+ TCM on B cells or plate-bound CD3/CD28/ICOS-L, CXCL13 secretion, as well as efficient induction of PC differentiation and Ig secretion, was associated with CXCR5 expression on TCM. Interestingly, these features were largely observed only for CXCR5+ TCM stimulated by B cells rather than either pDCs or cDCs. These results support the hypothesis that quick access to B cell follicles followed by cognate interaction with B cells induces efficient and preferential B cell helper function in CXCR5+ TCM over CXCR5+ TCM.

Discussion

The distinctive expression of the follicle-homing chemokine receptor CXCR5 on a subset of TCM suggested that these cells may represent a specialized subset of memory-type TFH cells. We therefore performed a detailed characterization of CXCR5+ TCM to determine their relationship with TFH cells and capacity to participate in and support B cell-related immune responses. CXCR5+ TCM did not exhibit the typical TFH cell phenotype ex vivo, or adopt it after in vitro activation under different stimulatory conditions. The CXCR5+ TCM did, however, acquire typical B cell helper characteristics to a greater extent than the corresponding CXCR5+ TCM cells. Most prominent was their ability to induce PC differentiation and Ig production, and secrete CXCL13. We attributed the distinct B cell helper qualities of CXCR5+ TCM mainly to their high expression of ICOS, their pronounced responsiveness to ICOS-L costimulation, and production of greater amounts of IL-10. Accordingly, neutralization of ICOS–ICOS-L interactions, and to a lesser extent IL-10, diminished PC differentiation and Ig secretion. The observation that B cell helper functions and CXCL13 secretion were only efficiently induced after stimulation by B cells, but not by DCs, further indicates that CXCR5+ TCM are preferentially recruited, over CXCR5+ TCM, to CXCL13-rich follicles. This would assure their preferential interaction with B cells, resulting in a quick and efficient protective humoral immune response.

In accordance with previous studies (18, 19, 32, 33), CXCR5+ TCM displayed a relatively unpolarized phenotype as compared with CXCR5+ TCM when examined ex vivo. They not only displayed a less pronounced Th1 and Th2, but also Th17/22 polarization (Figs. 2, 3; Supplemental Figs. 4–6), and expressed increased IL-10 and cMAF levels. This differently skewed phenotype supports the notion that CXCR5 expression on TCM defines a memory cell population with a potential for a specialized effector function to take part in humoral immune responses rather than other Th-lineage-specific effector functions. The lesser degree of polarization ex vivo as compared with CXCR5+ TCM was originally interpreted that CXCR5+ TCM might fall into a pool of CXCR5+ TCM might fall into a pool of CXCR5+ TCM. CXCR5+ TCM may be preferentially and quickly recruited to CXCL13-rich B cell follicles. Furthermore, their less polarized commitment to the Th1/2/17/22 lineages may facilitate their ability to efficiently become Tfh-like type cells, and thus focus on humoral rather than other inflammatory immune responses that may, in principle, be possible considering the plasticity of CD4 T cell lineage differentiation. In a recent study, Morita et al. (61) also concluded that human blood CXCR5+ CD4+ T cells appeared to represent the circulating memory counterpart of Tfh cells, and within this broad population they distinguished Th1-, Th2-, and Th17-like precursor subsets based on CXCR3 and CCR6 expression.

In our study, CXCR5+ TCM were especially distinguished by higher ICOS levels and a greater responsiveness to ICOS-L stimulation with significantly increased IL-10, IL-17, and CXCL13 secretion, whereas CD40L, Bcl-6, IL-21, and IFN-γ were more or less equally expressed by both subsets. Generally, IL-4 directs GC B cells into memory cells, whereas IL-10 typically steers them toward a PC phenotype (62–64). Also, IL-21 has recently been recognized as a potent PC differentiation factor in human B cells, rapidly generating plasmablasts from naive, memory, and GC B cells in vitro (41, 42, 65, 66). Furthermore, ICOS–ICOS-L interactions have been shown to play an important role in the differentiation of regulatory T cells (56, 59, 82–84), and the expression of ICOS and ICOS-L on TCM suggests that these cells may contribute to the induction and maintenance of TREG subsets (56, 59, 82–84).

For CD57, ICOS, PD1, and OX40, differences in the expression levels were observed (data not shown). For CD40L and of four independent donors for all other markers examined. For CD40L, expression levels, cells were additionally stimulated with PMA and ionomycin for 1 h before surface staining. Data represent mean percentage ± SD of three independent donors for CD40L and of four independent donors for all other markers examined. For CD57, ICOS, PD1, and OX40, differences in the expression levels ± SD of three independent donors for CD40L and of four independent donors for all other markers examined. For CD57, ICOS, PD1, and OX40, differences in the expression levels ± SD of three independent donors for CD40L and of four independent donors for all other markers examined. For CD57, ICOS, PD1, and OX40, differences in the expression levels ± SD of three independent donors for CD40L and of four independent donors for all other markers examined.
role in T-dependent Ab responses, Ig class switching, and GC formation (52, 67, 68), and engagement of ICOS on CD4 T cells superinduces IL-10 synthesis (69). In view of the comparable IL-21 expression by both CXCR5+ and CXCR5<sup>+</sup> T<sub>CM</sub>, and its strongest effects on Ig secretion by CD40L-stimulated B cells (Supplemental Fig. 9), we are convinced that IL-21 substantially contributes to the B cell helper function exerted by both CXCR5+ and CXCR5<sup>+</sup> T<sub>CM</sub>. Because IL-21 production was only slightly different between the two subsets, we propose that in CXCR5+ T<sub>CM</sub>, especially stronger ICOS–ICOS-L interactions, and to a lesser degree IL-10 secretion, most significantly contributed to their increased B cell helper function. This could be confirmed by FIGURE 5. Differential production of CXCL13 and increased levels of cMAF and ICOS<sup>hi</sup> by CXCR5<sup>+</sup> T<sub>CM</sub> after plate-bound in vitro stimulation. A, B, and E, Expression of Bcl-6 mRNA (A), CXCL13 mRNA (B), and ROR<gamma> and cMAF mRNA (E) determined by quantitative PCR of purified CXCR5<sup>+</sup> and CXCR5<sup>+</sup> T<sub>CM</sub> before and after in vitro stimulation for 48 h on anti-CD3/CD28– or anti-CD3/ICOS-L–coated plates. C, CXCL13 secretion determined by ELISA in culture supernatants of purified CXCR5<sup>+</sup> and CXCR5<sup>+</sup> T<sub>CM</sub> stimulated in vitro for 5 d with either anti-CD3/CD28– or anti-CD3/ICOS-L–coated plates. D, Expression of ICOS, CD40L, and CD57 as determined by flow cytometry after in vitro stimulation of purified CXCR5<sup>+</sup> and CXCR5<sup>+</sup> T<sub>CM</sub> for 48 h with either anti-CD3/CD28– or anti-CD3/ICOS-L–coated plates. Depicted are graphs with each symbol representing cells obtained from different donors. In addition, dot plots of ICOS and CD40L expression are shown for CXCR5<sup>+</sup> and CXCR5<sup>+</sup> T<sub>CM</sub> before and after stimulation. The rectangle in the representative dot plots (left and middle panels) marks CD40L/ICOS negative versus positive cells, whereas the gate in the right panels marks ICOS<sup>hi</sup> cells. A–E, Each symbol represents cells obtained from different donors. For stimulated cells, p value was calculated using paired t test; small horizontal bars indicate the grand mean.
FIGURE 6. Increased levels of ICOS expression and IL-10 secretion by CXCR5+ TCM account for their greater B cell helper function. A, Purified Vβ2+ CXCR5+ or CXCR5– TCM were incubated with autologous CD19+CD20+ B cells in the presence of TSST for 5 d; cells were then harvested, and expression levels of IL-21, IL-10, IL-4, IFN-γ, and IL-17 were determined by intracellular cytokine staining after restimulation with PMA and ionomycin for 5 h. Each symbol represents cells obtained from different donors; p value was calculated using paired t test; small horizontal bars indicate the grand mean. For IL-21 and IL-10 expression, dot plots are shown of one representative donor.

B and C, Purified Vβ2+ CXCR5+ or CXCR5– TCM were incubated with autologous CD19+CD20+ B cells in the presence of TSST. After 5–6 d of culture, CD3+ T cells were either resorted and expression of Bcl-6 and CXCL13 mRNA determined by quantitative PCR (B), or harvested and expression of ICOShi and CD57 determined by flow cytometry (C). Each symbol represents cells obtained from different donors; p value was calculated using paired t test; small horizontal bars indicate the grand mean. Also shown is CD57 expression on CXCR5+ and CXCR5– TCM before and after stimulation on B cells and ICOShi expression after stimulation on B cells (dot plots of one representative donor).

D and E, CD19+CD20+ B cells were incubated in the presence or absence of allogeneic purified CXCR5+ or CXCR5– TCM and neutralizing anti-IL-10 and anti–ICOS-L mAbs where indicated. After 6–7 d of culture, cells and supernatants were harvested. The frequency of B cells with a CD38hi CD20– phenotype was determined by flow cytometry, and the levels of secreted IgM, IgA, and IgG were measured by ELISA. D, Shown are Ig levels and
diminished PC differentiation and Ig secretion in the presence of neutralizing anti–ICOS-L and anti–IL-10 mAbs. The important role of high levels of ICOS expression on and strong responsiveness toward ICOS-L costimulation of CXCR5+ TCM is also reflected in their increased IL-10 secretion and especially strong induction of IgA secretion by B cells as compared with CXCR5− TCM. Our results differ somewhat from the study by Morita et al. (61), who concluded that IL-21 production was one of the main distinguishing features of CXCR5+ versus CXCR5− TCM.

In consideration of the fact that CXCR5+ TCM provide more efficient B cell help than CXCR5− TCM despite equal expression of Bcl-6, we assume that Bcl-6 expressed by CD4 T cells does not itself play a predominant role in this process. Further support for this assumption comes from Ma et al. (30), Rasheed et al. (29), and our own data, showing that tonsillar CD4 T cells that express intermediate levels of CXCR5, yet low levels of Bcl-6, were more efficient in inducing Ig secretion by cocultured B cells than T FH cells that express high levels of Bcl-6. Furthermore, effects of IL-17 and IFN-γ on Ig secretion in human B cells could be excluded (Supplemental Fig. 9). Interestingly, stimulation of CXCR5+ T CM on ICOS-L also resulted in a noticeable expression of the inflammatory cytokines IL-17 and IFN-γ, which was surprising to us in view of the unpolarized phenotype of these cells in their resting state. It is possible that slightly higher cMAF levels, measured in CXCR5+ T CM both ex vivo and after ICOS-L stimulation, resulted in increased IL-17 secretion. This would be consistent with cMAF, downstream of ICOS, regulating IL-21 production by Th17 and T FH cells (59), providing further reason to speculate that cMAF may indirectly contribute to B cell differentiation and Ig secretion via the induction of IL-21.

Dysregulation of T FH cell function may underpin certain types of autoimmune diseases. For instance, sanroque mice, which carry a mutation in the gene encoding the ICOS repressor roquin, develop a severe autoimmune lupus-like disease with accumulation of CXCR5+ICOShi TFH cells, GC formation, and autoantibody production (28, 70). Furthermore, an increased fraction of ICOS+ (71–73) and circulating CXCR5+ICOShi TFH-like cells (74) has been detected in the peripheral blood of systemic lupus erythematosus patients, most probably reflecting the dysregulated GC reaction, which correlates with titers of autoAbs and severity of end-organ involvement. Based on their prominent IL-17 secretion when stimulated in the presence of ICOS-L, their expression of high ICOS levels, and ability to promote humoral immune responses, we propose that CXCR5+ TCM perform specialized helper functions in inflammatory settings such as autoimmune diseases. Patients with juvenile dermatomyositis, a poorly understood systemic autoimmune disease, had expanded Th2-like and Th17-like CXCR5+ CD4+ T cells in the blood (61). These two subsets, in particular, may provide for excessive B cell help in this autoimmune disease. The enumeration and/or activation of CXCR5+ T CM in patients offers a new opportunity for a marker of disease activity, particularly in Ab-mediated autoimmune diseases.
One interesting feature of CXCR5+ TCM was its high expression and production of CXCL13. CXCL13 is required for the normal organization of B cell follicles in secondary lymphoid tissues (75), as well as ectopic lymphoid tissue neogenesis when overexpressed in extralymphoid sites (76). The function of ectopic lymphoid follicles remains incompletely defined, but they most probably support the pathogenesis of autoimmune diseases by contributing to the generation of high-affinity autoantibodies; in accordance with that, CXCL13 levels in autoantibody-mediated autoimmune diseases correlated with disease severity and progressive organization of tertiary lymphoid-like structures (77, 78). The observation that CXCL13 expression by infiltrating memory CD4 T cells is required for ectopic GC progression (77, 79) makes CXCR5+ TCM potential candidates to be recruited to these sites and perform their effector functions after adequate stimulation. These may include the support of humoral immune responses, the progression of the follicular response by CXCL13 secretion, and maintenance of the inflammatory milieu by secreting inflammatory cytokines. This hypothesis, as well as the still unclear question of which phenotype is adopted by CXCR5+ TCM after their recruitment to follicles, may ideally and properly be answered by means of future in vivo models. Even if some T FH cells may serve as a useful biomarker, for instance, to examine vaccination strategies or to stratify or monitor treatment of certain autoimmune or immunodeficiency patients.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Fig. 1. Isolation of CXCR5⁺ and CXCR5⁻ T_{CM} from MACS enriched CD4 T cells and sorted as CD4⁺CD45RA⁻CD25⁻CCR7⁺CXCR5⁺⁻ cells. Staining before and after sorting is shown.
Supplemental Fig. 2. Naïve CD20^+CD38^−CD27^− B cells were sorted from spleen mononuclear cells (n = 1 donor); naïve CD4^+CD45RA^+CXCR5^−, CD4^+CD45RA^−CXCR5^lo, CD4^+CD45RA^−CXCR5^int and CD4^+CD45RA^−CXCR5^hi T cells were sorted from tonsillar mononuclear cells (2 independent donors) and CXCR5^+ and CXCR5^− TCM were sorted from PBMCs (2 independent donors). The CD4 T cell subsets of 2 independent donors were separately incubated with equal amounts of sorted splenic naïve B cells from the same donor in the presence of anti-CD3/CD28 mAb. Ig secretion was determined in culture supernatants after 7d via ELISA. Displayed are mean ± SD of 2 independent donors.
**Supplemental Fig. 3.** CD20⁺CD19⁺ B cells were cultured with equal numbers of either allogeneic CXCR5⁺ or CXCR5⁻ T_CM and the number of surviving cells was quantitated after 5d of culture. Displayed are mean ± SD of three different experiments for B cells cultured in the presence of either CXCR5⁺ or CXCR5⁻ T_CM.
Supplemental Fig. 4. Freshly purified CXCR5+ and CXCR5− TCM isolated out of peripheral blood and TFH and naïve CD4 T cells isolated out of tonsils were examined for expression of GATA-3 mRNA, T-bet mRNA, RORγt mRNA, Bcl-6 mRNA and CXCL13 mRNA by quantitative PCR. Displayed are mean + SD of three independent donors.
Supplemental Fig. 5. Freshly purified CXCR5⁺ and CXCR5⁻ T<sub>CM</sub> isolated out of peripheral blood and T<sub>FH</sub> isolated out of tonsils were examined for the expression of co-stimulatory molecules by flow cytometry as indicated.
Supplemental Fig. 6. Freshly purified CXCR5+ and CXCR5− TCM isolated out of peripheral blood and TFH isolated out of tonsils were stimulated with PMA and ionomycin for 5h and expression of cytokines analyzed by flow cytometry as indicated.
Supplemental Fig. 7. Freshly purified CXCR5$^+$ and CXCR5$^-$ T$_{CM}$ were stimulated with anti-CD3 and PdBu and IL-10, IFN$_{\gamma}$, IL-17, IL-4 and IL-22 measured in 24h culture supernatants. Depicted are the results of one representative donor with experiments performed in duplicates and results presented as mean + SD.
Supplemental Fig. 8. Expression of IL-21 mRNA determined by quantitative PCR of purified CXCR5− and CXCR5+ TCM stimulated in vitro for 48h with either anti-CD3/CD28 or anti-CD3/ICOS-L coated plates. Each symbol represents cells obtained from different donors. P-value was calculated using paired Student’s t-test.
Supplemental Fig. 9. CD20^CD27^- naïve and CD20^CD27+ memory B cells were cultured for 12d in the presence of CD40L +/- IL-10, IL-17, IL-21 and IFNγ. Depicted are the results as mean ± SEM of one representative donor (n = 2).