Human tonsil *B-cell lymphoma 6* (*BCL6*)-expressing CD4⁺ T-cell subset specialized for B-cell help outside germinal centers

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T follicular helper (Tfh) cells represent a Th subset engaged in the help of B-cell responses in germinal centers (GCs). Tfh cells abundantly express the transcription repressor B-cell lymphoma 6 (*Bcl6*), a factor that is necessary and sufficient for their development in vivo. Whether Tfh or Tfh-committed cells are involved in the help of B cells outside GCs remains unclear, particularly in humans. In this study, we identified a previously undefined *BCL6*-expressing CD4⁺ T-cell subset in human tonsils. This subset expressed IL-7 receptor and chemokine receptor 5 (CXCR5) and inducible costimulator (ICOS) at low levels (CXCR5^{lo}ICOS^{lo}), and it was found exclusively outside GCs. CXCR5^{lo}ICOS^{lo} CD4⁺ T cells secreted larger amounts of IL-21 and IL-10 than CXCR5^{hi}ICOS^{hi} GC-Tfh cells upon activation, and they induced proliferation and differentiation of naïve B cells into Ig-producing cells more efficiently than GC-Tfh cells. However, this subset lacked the capacity to help GC-B cells because of the induction of apoptosis of GC-B cells through the FAS/FAS-ligand interaction. CXCR5^{lo}ICOS^{lo} CD4⁺ T cells expressed equivalent amounts of *BCL6* transcript with CXCR5^{hi}ICOS^{hi} GC-Tfh cells, but they expressed less Bcl6 protein. Collectively, our study indicates that CXCR5^{lo}ICOS^{lo} CD4⁺ T cells in human tonsils represent Tfh lineage-committed cells that provide help to naïve and memory B cells outside GCs.

CD4⁺ T cells provide a critical help to B cells to induce antibody responses. CD4⁺ T cells primed by dendritic cells (DCs) loaded with antigens interact with antigen-primed naïve B cells at extrafollicular sites (1), typically at the border of the T-cell zone and primary B-cell follicles (2). This interaction initiates the B-cell differentiation process to two different paths: extrafollicular plasma cells and cells forming germinal centers (GCs) (3–5). Extrafollicular plasma cells contribute to the early generation of specific antibodies after antigen challenge (6). GC-B cells subsequently differentiate into either high-affinity long-lived plasma cells or memory B cells after an extensive selection step (7, 8), and thus, they are central in the generation of B-cell memory.

T follicular helper (Tfh) cells have been recently established as a Th subset specialized for the help of B cells in GCs (9, 10). Whereas no specific markers are available to distinguish Tfh cells from other canonical Th subsets such as Th1, Th2, and Th17 cells, several molecules associated with the biological properties of Tfh cells are used for their identification. The chemokine (CXC) receptor 5 (CXCR5) expressed by Tfh cells (11–14) guides their migration into B-cell follicles in response to the ligand CXCL13 secreted by follicular dendritic cells (15). Tfh cells express programmed death-1 (PD-1), which was shown to play a role in the selection of high-affinity B cells in GCs (16). Inducible costimulator (ICOS) is also expressed by Tfh cells in human tonsils at high density (14). ICOS is critical for the development (17, 18) and functions (19, 20) of Tfh cells in both humans and mice. Studies in mice showed that overexpression of ICOS leads to the substantial increase of GC formation associated with exaggerated Tfh responses (21), which directly induce autoimmunity (22). Tfh cells also express other molecules required for interactions with B cells, including CD40 ligand (CD40L), CD84 (23), and signaling

lymphocyte activation molecule-associated protein (9). Compared with other canonical Th subsets, Tfh cells secrete larger amounts of IL-21 (24, 25), a γ c-family cytokine that strongly promotes the growth, differentiation, and class switching of B cells (26–31). Tfh cells express large amounts of the transcription repressor B-cell lymphoma 6 (*Bcl6*) (25). Mouse studies indicate that *Bcl6* positively regulates the generation of Tfh cells in vivo (32–34). In contrast, B lymphocyte-induced maturation protein 1 (Blimp-1), a transcription repressor that inhibits the function of *Bcl6*, negatively regulates Tfh development (34). Thus, the development of Tfh cells is regulated by the balance of these two molecules.

Whereas Tfh cells are considered to help the selection and differentiation of B cells in GCs, the identity of CD4⁺ T cells interacting with B cells outside GCs remains largely unknown. Studies with Murphy Roths Large (MRL)/*FAS*^{lpr} lupus-prone mouse models showed the presence of extrafollicular CD4⁺ T cells that display a similar phenotype as Tfh cells, such as up-regulation of *Bcl6* and IL-21 and down-regulation of P-selectin glycoprotein ligand 1 (35, 36). Whereas these studies show that extrafollicular helper cells share properties with Tfh cells under certain conditions in mice, it is unknown whether this finding holds true in humans.

Here, we identified a previously undefined *BCL6*-expressing CD4⁺ T-cell subset in human tonsils. This subset was identified by the expression of IL-7 receptor (IL-7R) and low levels of CXCR5 and ICOS (CXCR5^{lo}ICOS^{lo}), and it was exclusively localized outside GCs. CXCR5^{lo}ICOS^{lo} CD4⁺ T cells secreted large amounts of IL-21 and IL-10 and efficiently helped naïve and memory B cells. However, they lacked the capacity to help GC-B cells and induced the apoptosis of GC-B cells through the FAS/FAS-ligand (FAS-L) interaction. Thus, this tonsillar CD4⁺ T-cell subset is specialized to help B cells outside GCs.

Results

CXCR5^{lo}ICOS^{lo} CD4⁺ T Cells Are Localized Outside GC. Previous studies showed that human tonsillar Tfh cells in GCs coexpress ICOS and CXCR5 at high densities (14). In our study, we obtained pediatric tonsil samples (from children ages 3–12 y), which contained 34.8 ± 0.9% of CXCR5^{hi}ICOS^{hi} GC-Tfh cells among tonsillar CD4⁺ T cells (Fig. 1*A* and *B*, blue gate) (mean ± SEM, *n* = 20). Tonsillar CD4⁺ T cells included at least three

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other populations defined according to the expression of CXCR5 and ICOS: CXCR5^{hi}ICOS^{hi} (black gate; 21.4 ± 1.1%), CXCR5^{lo}ICOS^{lo} (red gate; 19.5 ± 0.9%), and CXCR5^{lo}ICOS^{hi} (green gate; 10.4 ± 0.5%) cells. As observed earlier (37, 38), a majority of CXCR5^{hi}ICOS^{hi} GC-Tfh cells expressed PD-1 (Fig. 1C) (84 ± 2%, mean ± SEM, *n* = 3). CXCR5^{lo}ICOS^{hi} CD4⁺ T cells also expressed PD-1, although at lower density than CXCR5^{hi}ICOS^{hi} GC-Tfh cells (41 ± 1%). Only a minor fraction of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells expressed PD-1 (8 ± 3%) at very low density. CD57, a molecule expressed by a fraction of human GC-Tfh cells (13), was expressed at high density by a fraction of CXCR5^{hi}ICOS^{hi} GC-Tfh cells (Fig. 1C) (35 ± 3%), whereas CXCR5^{lo}ICOS^{hi} CD4⁺ T cells and CXCR5^{lo}ICOS^{lo} CD4⁺ T cells contained less CD57-expressing cells (Fig. 1C) (10 ± 1% and 5 ± 1%, respectively). CXCR5^{hi}ICOS^{hi} CD4⁺ T cells did not express PD-1, CD57, or CD45RO, and thus, they were mostly composed of naïve CD4⁺ T cells. As reported previously (38, 39), a large fraction of CXCR5^{hi}ICOS^{hi} GC-Tfh cells did not express IL-7R (CD127) (Fig. 1C). In contrast, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells expressed IL-7R as much as CXCR5^{lo}ICOS^{hi} naïve CD4⁺ T cells, whereas CXCR5^{lo}ICOS^{hi} CD4⁺ T cells expressed IL-7R only at low density. A previous study reported the absence of IL-7R-expressing cells in GCs (39). We confirmed this observation by immunohistochemistry (Fig. 2A). Whereas both CXCR5^{hi}ICOS^{hi} and CXCR5^{lo}ICOS^{lo} CD4⁺ T cells expressed IL-7R, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells coexpressed CD45RO (Fig. 1C). Thus, the localization of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells was determined by analyzing IL-7R⁺CD45RO⁺CD4⁺ T cells by confocal microscopy. As shown in Fig. 2B and C, the cells coexpressing the three molecules (green, CD45RO; red, CD4; blue, IL-7R; white, triple positive cells) were exclusively localized outside GCs. Although we cannot exclude the possibility that the identified IL-7R⁺CD45RO⁺CD4⁺ T cells might contain

Fig. 1. Four Th populations in pediatric tonsil samples. (A) Four tonsillar Th populations were defined according to the expression of ICOS and CXCR5. (B) The frequency of each Th population (*n* = 20). One-way ANOVA Bonferroni multiple comparison test. ****P* < 0.001. (C) Expression of cell surface molecules (a representative from three experiments).

Fig. 2. CXCR5^{lo}ICOS^{lo} cells localize exclusively outside GCs. (A) Localization of IL-7R⁺ cells in tonsils was analyzed by immunohistochemistry using a frozen tonsil section. FM, follicular mantle. (Magnification: 4×.) (B and C) Localization of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells was analyzed by the coexpression of CD4 (red), CD45RO (green), and IL-7R (blue) by immunofluorescence microscopy (10×). Triple positive cells illustrated by the white color include CXCR5^{lo}ICOS^{lo} CD4⁺ T cells. A higher magnification view (40×) is shown in C (analyzed by confocal microscopy).

CXCR5^{lo}ICOS^{hi} CD4⁺ T cells, our observation shows that CXCR5^{lo}ICOS^{lo} CD4⁺ T cells are exclusively localized outside but not within GCs.

Tonsillar Th Populations Differentially Help B-Cell Subsets. We next assessed whether the four tonsillar Th populations were able to help the three major tonsillar B-cell subsets: naïve B (IgD⁺CD38[−]CD19⁺) cells, memory B (IgD[−]CD38⁺CD19⁺) cells, and GC-B (IgD[−]CD38⁺CD19⁺) cells (Fig. S1A). The tonsillar Th populations were carefully isolated by gating out cells expressing CD20, CD8, and CD56 during the sort. This strategy was particularly important for the isolation of CXCR5^{hi}ICOS^{hi} GC-Tfh cells, because 3–5% of CXCR5^{hi}ICOS^{hi} GC-Tfh cells coexpressed CD20 (Fig. S1B), likely representing conjugates of GC-Tfh and GC-B cells (40). The four tonsillar Th populations (2 × 10⁴/well) were cultured with each autologous B-cell subset (2 × 10⁴/well), and secreted Igs were measured at day 8 (Fig. 3A–C). To mimic the antigen-specific cognate interactions between B and T cells, staphylococcal enterotoxin B (SEB), a superantigen, was added to the cocultures. Previous studies showed that CXCR5^{hi}ICOS^{hi} GC-Tfh cells efficiently help tonsillar B cells

Fig. 3. Tonsillar Th populations differentially help B-cell subsets. (A–C) Ig production by B-cell subsets cocultured with tonsillar Th populations. (A) GC-B cells ($n = 11$ except CXCR5^{lo}ICOS^{hi}, which is $n = 3$), (B) naïve B cells ($n = 4$), and (C) memory B cells ($n = 6$ except CXCR5^{lo}ICOS^{hi}, which is $n = 3$) were cocultured with each Th population in the presence of SEB for 8 d. One-way ANOVA Bonferroni multiple comparison test. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. (D and E) Recovery of GC-B cells cocultured with Th populations. GC-B cells cultured for 8 d with each Th population were stained with CD3 and CD4 mAbs. Surface IgG and IgA expression by B cells cultured with CXCR5^{hi}ICOS^{hi} GC-Tfh cells is shown in *Right* (a representative from four experiments). Absolute number of viable GC-B cells per well (day 8) are shown in *E* ($n = 5$). One-way ANOVA Bonferroni multiple comparison test. (F) Recovery of T cells cocultured with GC-B cells. Absolute number of viable T cells per well (day 8; $n = 5$). One-way ANOVA Bonferroni multiple comparison test. (G) Proliferation and CD38 expression of CFSE-labeled naïve B cells cultured for 8 d with each Th population (a representative from three experiments). (H) Recovery of naïve B cells cocultured with Th populations. Absolute number of viable CD38⁺ plasmablasts per well (day 8; $n = 3$). One-way ANOVA Bonferroni multiple comparison test. (I) Recovery of T cells cocultured with naïve B cells. Absolute number of viable T cells per well (day 8; $n = 3$). One-way ANOVA Bonferroni multiple comparison test.

compared with other tonsillar Th populations (14, 24, 41). Consistently, CXCR5^{hi}ICOS^{hi} GC-Tfh cells (blue symbols) efficiently promoted GC-B cells to produce Igs (Fig. 3A) (IgM, $1.3 \pm 0.4 \mu\text{g/mL}$; IgG, $1.0 \pm 0.2 \mu\text{g/mL}$; IgA, $0.3 \pm 0.1 \mu\text{g/mL}$; mean $\pm$ SEM, $n = 11$). Other tonsillar Th populations, CXCR5^{lo}ICOS^{lo} cells (gray symbols), CXCR5^{lo}ICOS^{lo} cells (red symbols), and CXCR5^{lo}ICOS^{hi} cells (green symbols) barely induced GC-B cells to produce Igs (Fig. 3A). Indeed, only CXCR5^{hi}ICOS^{hi} GC-Tfh cells were able to maintain the survival of GC-B cells during the 8-d coculture (Fig. 3D and E), some of which expressed surface Igs (Fig. 3D *Right*). Notably, whereas GC-Tfh cells are prone to apoptosis because of expression of FAS (11, 14, 42), GC-B cells were able to maintain the survival of CXCR5^{hi}ICOS^{hi} GC-Tfh cells and induce their proliferation (Fig. 3F) ($142 \pm 14 \times 10^3$ cells/well, mean $\pm$ SEM, $n = 5$; 7.1 ± 0.7 -fold increase from the input). Thus, GC-Tfh and GC-B cells reciprocally help each other.

In contrast, upon coculture with naïve B cells, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells were the most efficient at inducing naïve B cells to produce IgM (Fig. 3B) ($33.0 \pm 4.9 \mu\text{g/mL}$, mean $\pm$ SEM, $n = 4$) as well as IgG and IgA (IgG, $1.1 \pm 0.2 \mu\text{g/mL}$; IgA, $0.8 \pm 0.2 \mu\text{g/mL}$). This finding was most due to a contamination of CD27⁺ antigen-experienced subepithelial B cells (43) (3–7% in IgD⁺CD38⁺CD19⁺ B cells), because CXCR5^{lo}ICOS^{lo} CD4⁺ T cells efficiently induced IgD⁺CD27⁺CD38⁺ highly purified naïve B cells to produce Igs (Fig. S1C). Furthermore, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells induced a robust proliferation of naïve B cells (Fig. 3G) and yielded more B cells expressing CD38,

a marker of plasmablasts, than other tonsillar Th populations (Fig. 3G and H). CXCR5^{hi}ICOS^{hi} GC-Tfh cells were capable of inducing naïve B cells to produce Igs but only at low amounts (Fig. 3B) (IgM, $1.6 \pm 0.5 \mu\text{g/mL}$; IgG, $0.2 \pm 0.1 \mu\text{g/mL}$; IgA, $0.1 \pm 0.1 \mu\text{g/mL}$; mean $\pm$ SEM, $n = 4$). This finding might partly be due to the poor survival of CXCR5^{hi}ICOS^{hi} GC-Tfh cells, because naïve B cells did not support their survival and/or proliferation in culture (Fig. 3I) ($29 \pm 8 \times 10^3$ cells/well, mean $\pm$ SEM, $n = 3$; 1.5 ± 0.4 -fold increase from the input). CXCR5^{lo}ICOS^{lo} CD4⁺ T cells and CXCR5^{lo}ICOS^{hi} CD4⁺ T cells were virtually unable to induce naïve B cells to produce Igs (Fig. 3B).

Upon coculture with memory B cells, both CXCR5^{lo}ICOS^{lo} CD4⁺ T and CXCR5^{hi}ICOS^{hi} GC-Tfh cells were equally efficient at inducing their Ig production (Fig. 3C). CXCR5^{lo}ICOS^{lo} cells and CXCR5^{lo}ICOS^{hi} CD4⁺ T cells barely induced memory B cells to produce Igs.

Thus, tonsillar Th populations differentially help B-cell subsets. CXCR5^{lo}ICOS^{lo} cells and CXCR5^{lo}ICOS^{hi} CD4⁺ T cells barely help any B-cell subsets. Whereas CXCR5^{hi}ICOS^{hi} GC-Tfh cells efficiently help GC-B cells, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells are the most potent at inducing naïve B cells to differentiate into Ig-producing cells.

Tonsillar Th Populations Differentially Secrete Cytokines/Chemokines. Tfh cells secrete IL-4, IL-10, and IL-21, each of which differentially regulates B-cell proliferation, differentiation, and class switching (9, 10). Thus, we analyzed whether the cytokine production profiles of these tonsillar Th populations explain their

differential effects on B-cell subsets. Upon coculture with naïve B cells, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells (Fig. 4A, red symbols in IL-21 plot) secreted the largest amounts of IL-21 (3.3 ± 0.4 ng/mL, mean $\pm$ SEM, $n = 6$). This finding held true when tonsillar Th populations were cultured with GC-B cells (Fig. 4B, IL-21 plot) (1.8 ± 0.4 ng/mL, $n = 5$), or stimulated through CD3/CD28 (Fig. S24) (6.1 ± 0.6 ng/mL, $n = 3$). CXCR5^{hi}ICOS^{hi} GC-Tfh cells also secreted IL-21 but at substantially lower amounts (Fig. 4A and B and Fig. S24) (culture with naïve B cells: 0.5 ± 0.2 ng/mL, $n = 6$; culture with GC-B cells: 0.4 ± 0.2 ng/mL, $n = 5$; CD3/CD28 stimulation: 0.7 ± 0.1 ng/mL, $n = 3$). The largest amounts of IL-10 were also detected in the cultures of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells with B cells (Fig. 4A and B) (with naïve B cells: 1.1 ± 0.2 ng/mL, $n = 6$; with GC-B cells: 1.4 ± 0.4 ng/mL, $n = 5$) as well as upon CD3/CD28 stimulation (Fig. S24). In contrast, consistent with previous studies (14, 25, 44), CXCR5^{hi}ICOS^{hi} GC-Tfh cells produced the largest amounts of CXCL13, a chemokine secreted by Tfh cells upon culture with B cells (Fig. 4C) (with naïve B cells: 2.7 ± 0.3 ng/mL, $n = 4$; with GC-B cells: 2.5 ± 0.2 ng/mL, $n = 3$). IL-4 was detected only at low concentrations (<0.3 ng/mL) in any Th cultures with B cells (Fig. 4A and B, IL-4 plot), although large amounts of IL-4 were secreted by CXCR5^{lo}ICOS^{lo} CD4⁺ T cells and CXCR5^{hi}ICOS^{hi} GC-Tfh cells upon CD3/CD28 stimulation (Fig. S24). CXCR5^{lo}ICOS^{lo} CD4⁺ T cells (green symbols) barely secreted IL-21 and IL-10 upon encounter with B cells (Fig. 4A and B) (with naïve B cells: 0.2 ± 0.0 ng/mL and 0.2 ± 0.0 ng/mL, respectively, $n = 6$; with GC-B cells: 0.4 ± 0.2 ng/mL and 0.2 ± 0.0 ng/mL, respectively, $n = 5$) but secreted the largest amounts of IL-17A (with naïve B cells: 4.1 ± 1.3 ng/mL; with GC-B cells: 4.3 ± 1.6 ng/mL).

Secretion of the largest amounts of IL-21 by CXCR5^{lo}ICOS^{lo} CD4⁺ T cells was somewhat unexpected, because a number of previous studies showed that GC-Tfh cells expressed the highest levels of IL-21 among CD4⁺ T-cell subsets (14, 24, 25, 32, 38). These conclusions were based on the analysis of *IL21* transcript by mRNA microarray or RT-PCR (14, 25, 38) or on the analysis of intracytoplasmic IL-21 expression after phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation (32, 38). Consistently, when isolated, CXCR5^{hi}ICOS^{hi} GC-Tfh cells expressed the most abundant *IL21* transcript among tonsillar CD4⁺ T-cell subsets (Fig. 4D, day 0) and the highest level of intracytoplasmic IL-21 on 6-h stimulation with PMA and ionomycin (Fig. S2B). The discrepancy between these observations and the secretion of IL-21 (Fig. 4A and B) might reflect differences in the kinetics of *IL21* expression upon activation. Therefore, we analyzed *IL21* expression by real-time RT-PCR at different time points after CD3/CD28 mAbs stimulation. Whereas *IL21* was expressed at high levels by isolated CXCR5^{hi}ICOS^{hi} GC-Tfh cells upon isolation (day 0), CXCR5^{hi}ICOS^{hi} GC-Tfh cells rapidly lost *IL21* expression after CD3/CD28 stimulation (Fig. 4D, day 1). In contrast, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells strongly up-regulated *IL21* expression in response to the stimulation. Loss of *IL21* by CXCR5^{hi}ICOS^{hi} GC-Tfh cells was not because of cell death, because recovery of viable T cells at day 1 was comparable between CXCR5^{lo}ICOS^{lo} CD4⁺ T cells and CXCR5^{hi}ICOS^{hi} GC-Tfh cells (CXCR5^{lo}ICOS^{lo} CD4⁺ T-cell recovery, $68 \pm 6\%$ of input; CXCR5^{hi}ICOS^{hi} GC-Tfh cell recovery, $70 \pm 9\%$ of input, mean $\pm$ SEM, $n = 3$). Furthermore, expression of *CXCL13* transcript by CXCR5^{hi}ICOS^{hi} GC-Tfh cells was maintained for at least 2 d after stimulation.

Fig. 4. Cytokine secretion profiles of tonsillar Th populations. (A and B) Cytokine secretion on interaction with B-cell subsets. The four Th populations were cultured with either naïve B cells (A; $n = 6$) or GC-B cells (B; $n = 5$), and the secretions of IL-10, IL-21, IL-17A, and IL-4 were analyzed on day 2. One-way ANOVA Bonferroni multiple comparison test. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. (C) CXCL13 secretion on interaction with naïve B cells (Upper; $n = 4$) or GC-B cells (Lower; $n = 3$). One-way ANOVA Bonferroni multiple comparison test. (D) Kinetics of *IL21* and *CXCL13* expression. The four Th populations were analyzed for the expression of *IL21* and *CXCL13* by real-time RT-PCR before and after stimulation with CD3/CD28 mAb-coated beads ($n = 2-3$). One-way ANOVA Bonferroni multiple comparison test for $n = 3$ and paired t test for $n = 2$.

Thus, the four tonsillar Th populations display distinct cytokine/chemokine secretion profiles. Upon interaction with B cells, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells secrete largest amounts of IL-10 and IL-21, whereas CXCR5^{hi}ICOS^{hi} GC-Tfh cells secrete largest amounts of CXCL13. CXCR5^{lo}ICOS^{hi} CD4⁺ T cells secrete the largest amounts of IL-17A.

Factors Involved in the Help of Naïve B Cells by CXCR5^{lo}ICOS^{lo} CD4⁺ T Cells. We next analyzed the molecules that were involved in the help of B-cell subsets by tonsillar Th populations. First, to test the role of IL-21 secreted by CD4⁺ T cells, a neutralizing IL-21R-Fc chimeric protein was added to the T- and B-cell cocultures. This analysis resulted in the complete inhibition of Ig production in all of the cultures of tonsillar Th populations and B-cell subsets, including those cultures of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells and naïve B cells and CXCR5^{hi}ICOS^{hi} GC-Tfh and GC-B cells (Fig. 5A). Second, the role of ICOS was tested by blocking the ICOS/ICOS-ligand (ICOS-L) interaction with an ICOS-blocking mAb. Inhibition of the ICOS/ICOS-L interaction completely abrogated Ig production by both naïve B and GC-B cells (Fig. 5B) as well as cytokine secretion by CD4⁺ T cells (Fig. 5C). Third, blocking IL-10 by using a combination of blocking mAbs (i.e., anti-IL-10 and anti-IL-10 receptor) resulted in a partial inhibition of Ig production in the cultures of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells with naïve B cells and CXCR5^{hi}ICOS^{hi} GC-Tfh cells with GC-B cells (Fig. S3). Last, blocking CD40L, a fundamental molecule for B-cell help, was analyzed. Neither CXCR5^{lo}ICOS^{lo} CD4⁺ T cells nor CXCR5^{hi}ICOS^{hi} GC-Tfh cells expressed CD40L when isolated, but both cells rapidly expressed CD40L upon activation (Fig. S4). Blocking CD40L also resulted in a partial inhibition of Ig production (Fig. S5). At

variance with blocking the ICOS/ICOS-L interaction, blocking the CD40/CD40L interaction did not inhibit the cytokine secretion from CD4⁺ T cells (Fig. 5C).

Thus, both CXCR5^{lo}ICOS^{lo} CD4⁺ T cells and CXCR5^{hi}ICOS^{hi} GC-Tfh cells help B-cell subsets in a fashion dependent on IL-21, ICOS, IL-10, and CD40L. Whereas the ICOS/ICOS-L interaction was essential for T-cell activation, the CD40/CD40L interaction was important for B-cell differentiation.

CXCR5^{lo}ICOS^{lo} CD4⁺ T Cells Induce Apoptosis of GC-B Cells Through the FAS/FAS-L Interaction. We were intrigued by the ability of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells to help both naïve and memory B cells, while being unable to help GC-B cells. Given that the recovery of viable T cells was similar between CXCR5^{lo}ICOS^{lo} CD4⁺ T cells and CXCR5^{hi}ICOS^{hi} GC-Tfh cells cultured with GC-B cells (Fig. 3F), the lack of help from GC-B cells did not seem to be because of total consumption of nutrients in cultures. Furthermore, titrating down the number of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells did not result in better help from GC-B cells (Fig. S6). Considering that cocultures of GC-B cells and CXCR5^{lo}ICOS^{lo} CD4⁺ T cells resulted in few remaining viable GC-B cells (Fig. 3E), we hypothesized that these T cells might induce the apoptosis of GC-B cells, which express high amounts of FAS (45–47) (Fig. 6A). Indeed, soluble FAS-L was detected in cocultures of GC-B cells with CXCR5^{lo}ICOS^{lo}, CXCR5^{lo}ICOS^{hi}, and CXCR5^{hi}ICOS^{hi} CD4⁺ T cells but not with CXCR5^{hi}ICOS^{hi} GC-Tfh cells (Fig. 6B). Consistently, *FASLG* transcript (encoding FAS-L) was expressed by the three tonsillar Th populations but not CXCR5^{hi}ICOS^{hi} GC-Tfh cells (Fig. 6C). Upon blocking the FAS/FAS-L interaction with a neutralizing anti-FAS antibody, CXCR5^{lo}ICOS^{lo} and CXCR5^{lo}ICOS^{hi} CD4⁺ T cells were

Fig. 5. Molecules associated with helper activity of tonsillar Th populations. (A) IL-21R/Fc chimera protein was added to the cultures of Th populations and the indicated B-cell subsets. Ig concentrations at day 8. A representative from four experiments. Paired *t* test. ****P* < 0.001, ***P* < 0.01, and **P* < 0.05. (B) ICOS mAb was added to the cultures of Th populations and the indicated B cells. Ig concentrations at day 8 (a representative from four experiments). Paired *t* test. (C) Cytokine secretion. CD40L mAb, ICOS mAb, or an isotype control was added to the cultures of Th populations and the indicated B cells. Cytokine concentrations at day 2 (a representative from three experiments).

Fig. 6. CXCR5^{lo}ICOS^{lo} CD4⁺ T cells induce apoptosis of GC-B cells through the FAS/FAS-L interaction. (A) Expression of cell surface FAS by GC-B cells. (B) Soluble FAS-L production by Th populations cocultured with GC-B cells. FAS-L concentrations at day 2 ($n = 4$). One-way ANOVA Bonferroni multiple comparison test. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. (C) *FASLG* mRNA expression by tonsillar Th populations analyzed by real-time RT-PCR. Expression of *FASLG* transcript was normalized to that of *HPRT1* transcript ($n = 3$). (D) Ig secretion by GC-B cells cocultured with tonsillar Th populations in the presence of FAS mAb. Ig concentrations were measured at day 8 (a representative from four experiments). (E) Ig secretion by naive B cells cocultured with CXCR5^{lo}ICOS^{lo} CD4⁺ T cells, CXCR5^{lo}ICOS^{hi} CD4⁺ T cells, or CXCR5^{hi}ICOS^{hi} GC-Tfh cells supplemented with titrated doses of recombinant IL-21. Ig secretion measured at day 8 (a representative from two experiments).

able to maintain the survival of GC-B cells (Fig. S7) and induce them to produce Igs (Fig. 6D). Thus, FAS-L is expressed by CXCR5^{lo}ICOS^{lo} and CXCR5^{lo}ICOS^{hi} CD4⁺ T cells but not CXCR5^{hi}ICOS^{hi} GC-Tfh cells, and it induces the apoptosis of FAS-expressing GC-B cells.

We next hypothesized that the differential capacity to help naive B cells among tonsillar Th populations depends on the capacity to produce IL-21. Thus, we examined whether supple-

mentation of IL-21 into the cocultures of tonsillar Th populations and naive B cells results in the secretion of higher amounts of Igs. As shown in Fig. 6E, IL-21 dose-dependently induced naive B cells to produce Igs when cocultured with CXCR5^{lo}ICOS^{hi} CD4⁺ T cells and CXCR5^{hi}ICOS^{hi} GC-Tfh cells. Thus, the difference in the capacity to help naive B cells among tonsillar Th subsets is at least partly dependent on their capacity to produce IL-21.

Thus, the superior capacity of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells to secrete IL-21 partly explains their capacity to provide efficient help to naive B cells.

CXCR5^{lo}ICOS^{lo} CD4⁺ T Cells Express Large Amounts of *BCL6* Transcript.

Recent studies in mice indicate that development of Tfh cells is reciprocally regulated by the two transcription repressors, Bcl6 and Blimp-1 (32–34). To determine the extent of Tfh lineage commitment, the expression of Bcl6 and Blimp-1 (encoded by *BCL6* and *PRDM1* genes, respectively) in tonsillar Th populations was analyzed by real-time RT-PCR. As shown in Fig. 7A, *BCL6* transcript was abundantly expressed by CXCR5^{lo}ICOS^{lo} CD4⁺ T cells compared with CXCR5^{lo}ICOS^{lo} naive CD4⁺ T cells, and the expression was equivalent to that of CXCR5^{hi}ICOS^{hi} GC-Tfh cells. The expression of *PRDM1* transcript was equally low between CXCR5^{lo}ICOS^{lo} CD4⁺ T cells and CXCR5^{hi}ICOS^{hi} GC-Tfh cells. In contrast, CXCR5^{lo}ICOS^{hi} expressed very high amounts of *PRDM1* transcript but little *BCL6* transcript.

Because Bcl6 expression is controlled by many post-transcriptional regulatory mechanisms (48), the expression of Bcl6 and Blimp-1 proteins were analyzed by Western blot. As shown in Fig. 7B, CXCR5^{hi}ICOS^{hi} GC-Tfh cells expressed more abundant Bcl6 protein than CXCR5^{lo}ICOS^{lo} CD4⁺ T cells. Consistent results were obtained by flow cytometry (Fig. 7C). Blimp-1 protein was barely detected in any tonsillar Th populations. We assumed that the difference in the expression of Bcl6 between CXCR5^{hi}ICOS^{hi} GC-Tfh cells and CXCR5^{lo}ICOS^{lo} CD4⁺ T cells might reflect the difference in the maturation stages. Indeed, whereas the expression of ICOS and PD-1 was low when isolated (Fig. 1C), CXCR5^{lo}ICOS^{lo} CD4⁺ T cells robustly up-regulated the expression of ICOS and PD-1 on interaction with naive B cells at even higher density than CXCR5^{hi}ICOS^{hi} GC-Tfh cells (Fig. 7D).

Thus, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells express large amounts of *BCL6* transcript but less Bcl6 protein than CXCR5^{hi}ICOS^{hi} GC-Tfh cells.

Frequency of CXCR5^{lo}ICOS^{lo} CD4⁺ T Cells Is Different in Adult Tonsils.

We analyzed whether CXCR5^{lo}ICOS^{lo} CD4⁺ T cells were also present in adult tonsils and human spleen. Whereas pediatric tonsil samples contained significantly more CXCR5^{hi}ICOS^{hi} GC-Tfh cells than CXCR5^{lo}ICOS^{lo} CD4⁺ T cells (Fig. 1B), the frequency of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells was slightly higher than that of CXCR5^{hi}ICOS^{hi} GC-Tfh cells in adult tonsil samples (Fig. 8A and B) (CXCR5^{lo}ICOS^{lo} CD4⁺ T cells, $32 \pm 5\%$; CXCR5^{hi}ICOS^{hi} GC-Tfh cells, $22 \pm 3\%$). In spleen, the majority of CD4⁺ T cells were CXCR5^{lo}ICOS^{lo}CD127⁺ CD4⁺ T cells, and CXCR5^{hi}ICOS^{hi} GC-Tfh cells were barely found. The separation between CXCR5^{lo}ICOS^{lo} and CXCR5^{lo}ICOS^{lo} CD4⁺ T-cell populations was not clear, and CXCR5^{lo}ICOS^{lo} CD4⁺ T cells, if there were any of these cells, expressed only low levels of CXCR5 (Fig. 8A).

Thus, the frequency of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells is different among human secondary lymphoid organs, and the CXCR5^{lo}ICOS^{lo} CD4⁺ T-cell population was not clearly identified in spleen.

Discussion

Our study characterizes a previously undefined *BCL6*-expressing human tonsillar CD4⁺ T-cell subset, which can be identified as CXCR5^{lo}ICOS^{lo}IL-7R⁺CD45RO⁺ cells. The abundance of

Fig. 7. CXCR5^{lo}ICOS^{lo} CD4⁺ T cells express large amounts of *BCL6*. (A) Expression of *BCL6* and *PRDM1* transcripts by tonsillar Th populations was analyzed by real-time RT-PCR. Expression of each transcript was normalized to that of *HPRT1* transcript ($n = 3$). One-way ANOVA Bonferroni multiple comparison test. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. (B) Expression of Bcl6 and Blimp-1 proteins in tonsillar Th populations. Sorted Th populations were lysed, and equal amounts of protein were loaded per well to analyze the expression of Bcl6 and Blimp-1 by Western blotting. For the positive controls, the lysate of GC-B cells was used for Bcl6 detection, and BJAB (a B-cell line) cell lysate was used for Blimp-1 detection. Expected Blimp-1 band is indicated with an arrow. Densitometry score ratio for Bcl6 and Blimp-1 against control actin protein is indicated in number (a representative of four experiments). (C) Analysis of Bcl6 expression in tonsillar CD4⁺ Th populations by flow cytometry (a representative of four experiments). (D) Up-regulation of ICOS and PD-1. The four Th populations were cultured with naïve B cells, and the expression of ICOS and PD-1 on T cells was analyzed at day 5 (a representative from two experiments).

BCL6 and *PRDM1* transcripts in CXCR5^{lo}ICOS^{lo} CD4⁺ T cells was similar to that in CXCR5^{hi}ICOS^{hi} GC-Tfh cells. A previous report showed that tonsillar CXCR5^{lo} CD4⁺ T cells express lower amounts of *BCL6* transcript than CXCR5^{hi} Tfh cells (38). The discrepancy between that study and our study lies in the difference in cell isolation strategies. Our study also separated the CXCR5^{lo} Th cell population into two populations according to the expression of ICOS. Whereas CXCR5^{lo}ICOS^{lo} CD4⁺ T cells expressed abundant *BCL6* transcript, CXCR5^{lo}ICOS^{hi} CD4⁺ T cells barely expressed *BCL6* transcript but expressed large amounts of *PRDM1* transcript. Thus, our study usefully complements this earlier study and shows that CXCR5^{lo} CD4⁺ T cells contain two

cell types with different functions: Tfh-committed CXCR5^{lo}ICOS^{lo} and non-Tfh-committed CXCR5^{lo}ICOS^{hi} cells. Indeed, CXCR5^{lo}ICOS^{hi} CD4⁺ T cells were poorly efficient at helping B-cell responses.

The two *BCL6*-expressing tonsillar Th subsets, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells and CXCR5^{hi}ICOS^{hi} GC-Tfh cells, were found to differentially help B cells. Consistent with previous studies showing efficient help of B cells by GC-Tfh cells (14, 24, 41), CXCR5^{hi}ICOS^{hi} GC-Tfh cells were efficient at helping GC-B cells. Reciprocally, GC-B cells were able to maintain the survival of CXCR5^{hi}ICOS^{hi} GC-Tfh cells, because the recovery of viable CXCR5^{hi}ICOS^{hi} GC-Tfh cells was significantly better

Fig. 8. CXCR5^{lo}ICOS^{lo} CD4⁺ T cells in adult tonsils. The four Th populations in pediatric tonsil samples, adult tonsil samples, and human spleen samples. The same number of T-enriched cells (after removing B cells with magnetic beads) was stained and analyzed in parallel in identical conditions. The four Th populations were determined based on the analysis of pediatric tonsil samples, and the same gates were applied for the adult and spleen samples. (A) A representative of at least two experiments gated to CD3⁺CD4⁺ T cells. (B) The frequency of each Th population in adult tonsils ($n = 3$) and spleen ($n = 2$). One-way ANOVA Bonferroni multiple comparison test for adult tonsil samples. * $P < 0.05$.

when cultured with GC-B cells than with naïve B cells (Fig. 3 *F* and *I*) ($P < 0.05$, $n = 3$, paired *t* test). Equivalent concentrations of cytokines were detected when CXCR5^{hi}ICOS^{hi} GC-Tfh cells were cultured with either GC-B or naïve B cells (Fig. 4 *A* and *B*), suggesting that the better survival of GC-Tfh cells does not depend on cytokines derived from GC-Tfh cells but rather, on intrinsic factors of GC-B cells that remain to be determined.

We found that CXCR5^{lo}ICOS^{lo} CD4⁺ T cells were far more efficient than GC-Tfh cells at inducing naïve B cells to proliferate and differentiate into Ig-producing cells. The difference between the two Th populations in the ability to help naïve B cells was at least partly explained by the differences in the secretion of cytokines, particularly IL-21. Our study shows that the two Th populations display intrinsically distinct cytokine production properties. Upon activation, GC-Tfh cells secreted substantially higher levels of CXCL13 protein and maintained higher levels of *CXCL13* transcript expression than CXCR5^{lo}ICOS^{lo} CD4⁺ T cells, showing that GC-Tfh cells display a machinery to produce large amounts of CXCL13. In contrast, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells secreted larger amounts of IL-10 and IL-21 than GC-Tfh cells upon stimulation with CD3/CD28 stimulation and even upon coculture with B cells. Whereas GC-Tfh cells express highest levels of *IL21* transcript upon isolation, GC-Tfh cells rapidly lost *IL21* expression upon activation. This finding was in contrast to the up-regulation of *IL21* transcript by CXCR5^{lo}ICOS^{lo} CD4⁺ T cells after activation, which leads to the secretion of large amounts of IL-21.

Our study suggests that CXCR5^{lo}ICOS^{lo}CD4⁺ T cells are committed to the Tfh lineage, because (*i*) they showed similar profiles of *BCL6* and *PRDM1* expression with GC-Tfh cells, (*ii*) they helped B cells in a manner dependent on IL-21, IL-10, ICOS, and CD40L, thus sharing functions with GC-Tfh cells, and (*iii*) they robustly up-regulated ICOS and PD-1 expression upon activation, thus sharing phenotype with GC-Tfh cells. CXCR5^{lo}ICOS^{lo}CD4⁺ T cells were exclusively localized outside GCs and therefore, might represent extrafollicular helper cells engaged in inducing the differentiation of B cells into extrafollicular plasma cells. Consistently, recent studies using lupus-prone mice showed that extrafollicular helper cells share the property with GC-Tfh cells, including Bcl-6 expression (35, 36). We cannot exclude, however, the possibility that CXCR5^{lo}ICOS^{lo} CD4⁺ T cells represent precursors of GC-Tfh cells. CXCR5^{lo}ICOS^{lo} CD4⁺ T cells expressed lower amounts of Bcl6 protein than CXCR5^{hi}ICOS^{hi} GC-Tfh cells, which might reflect the fact that CXCR5^{lo}ICOS^{lo} CD4⁺ T cells are in a transition stage of differentiating into mature GC-Tfh cells. Robust up-regulation of ICOS and PD-1 by CXCR5^{lo}ICOS^{lo} CD4⁺ T cells upon interaction with B cells supports this hypothesis. Alternatively, CXCR5^{lo}ICOS^{lo} CD4⁺ T cells might constitute both extrafollicular helper cells and GC-Tfh precursors and modify their balance according to the microenvironment of secondary lymphoid organs.

The specialized role of CXCR5^{lo}ICOS^{lo}CD4⁺ T cells for the help of B cells outside GCs was also supported by the observation that they lacked the capacity to help GC-B cells. Instead, they induced the apoptosis of GC-B cells through the interaction of FAS/FAS-L. Whereas activated naïve B cells and memory B cells also express FAS (45), CXCR5^{lo}ICOS^{lo} CD4⁺ T cells did not induce their apoptosis, likely because naïve and memory B cells express abundant Bcl2, which protects them from apoptosis through the FAS/FAS-L interaction (45). Induction of apoptosis of GC-B cells by CXCR5^{lo}ICOS^{lo} CD4⁺ T cells might represent a fail-safe system to eliminate GC-B cells that prematurely migrate out of GCs. Alternatively, because the FAS/FAS-L interaction is essential for the depletion of low-affinity GC-B cells during GC reactions (49), CXCR5^{lo}ICOS^{lo} CD4⁺ T cells that have just migrated into GCs, provided that they have an ability to do so, might be involved in this type of negative selection process

of B cells in GCs. In addition, CXCR5^{lo}ICOS^{hi} CD4⁺ T cells, which largely lacked an ability to help B cells, were also able to help GC-B cells when the FAS/FAS-L interaction was blocked. Thus, CXCR5^{lo}ICOS^{hi} CD4⁺ T cells might also be involved in this negative selection process. In line with this finding, CXCR5^{lo}ICOS^{hi} CD4⁺ T cells contained CD57⁺ cells, which were reported to express FAS-L (42).

Identification of *BCL6*-expressing Tfh-committed cells outside GCs suggests that the differentiation of human Tfh cells initiates outside B-cell follicles. Therefore, similar to other conventional Th subsets, the Th differentiation program to the Tfh lineage might initiate when naïve CD4⁺ T cells interact with antigen-presenting DCs. This observation supports our previous proposal that a particular type of DC subset is specialized for the development of Tfh cells (50). In humans, IL-12 secreted by DCs seems to be a major cytokine in the development of IL-21-producing Tfh-like cells (51, 52). Furthermore, IL-12 was shown to induce Bcl6 expression in human CD4⁺ T cells (38, 53). Therefore, IL-12-producing DC subsets, such as CD14⁺ dermal DCs (50), likely represent an important human DC subset that initiates the development of Tfh cells.

In conclusion, tonsillar CXCR5^{lo}ICOS^{lo} CD4⁺ T cells represent Tfh-committed cells helping B-cell responses outside GCs. Determining whether circulating CXCR5⁺ CD4⁺ T cells, a cell population proposed to be a memory component of Tfh cells (54, 55), can be derived from CXCR5^{lo}ICOS^{lo} CD4⁺ T cells will be of great interest. Additional characterization of CXCR5^{lo}ICOS^{lo} CD4⁺ T cells together with determination of their developmental mechanisms will bring significant insights to the understanding of human autoimmune disease pathogenesis, in which extrafollicular plasma cell differentiation might be involved (36). Furthermore, studies on CXCR5^{lo}ICOS^{lo}CD4⁺ T cells will be also beneficial for the design of vaccines against infectious diseases.

Materials and Methods

Cell Isolation. All of the studies described here were approved by the Institutional Review Board of Baylor Research Institute. Tonsil samples were obtained from young patients (3–12 y) or adults undergoing tonsillectomy, and single cells were collected by mechanical disruption of tonsil samples. Spleen samples were obtained from a cadaveric organ donor or the tissue bank at Baylor University Medical Center. B cells were first positively selected with CD19 MACS Microbeads (Miltenyi Biotec). For the isolation of Th populations, the CD19-negative fraction was stained with biotin-conjugated anti-ICOS (ISA-3; eBioscience) /streptavidin PE-Cy7 (BD), anti-CD20 FITC (2H7; eBioscience), anti-CXCR5 phycoerythrin (PE) (51505.111; R&D Systems), anti-CD4 Pacific Blue (RPA-T4; BD), anti-CD8 allophycocyanin (APC) (RPA-8; eBioscience), and anti-CD56 APC (MEM 188; eBioscience). The four Th populations were sorted with FACSARIA (BD Biosciences) according to the expression of CXCR5 and ICOS within the CD4⁺CD20[−]CD8[−]CD56[−] cell population. For the isolation of B-cell subsets, CD19⁺ B cells were stained with anti-CD20 FITC (2H7; eBioscience), anti-CD3 PE-Cy5 (UCHL1; eBioscience), anti-IgD PE (IA6-2; BD), and anti-CD38 APC (HIT2; eBioscience). Naïve B cells (CD20⁺IgD[−]CD38[−]), memory B cells (CD20⁺IgD[−]CD38⁺), and GC-B cells (CD20⁺IgD[−]CD38⁺) were sorted within the CD3-negative cell population.

Phenotype Analysis. The phenotypes of tonsillar Th and B-cell subsets were analyzed with the antibodies anti-PD1 PE (J116; eBioscience), CD57 PE (NK-1; SouthernBiotech), CD45RO PE-Cy5 (UCHL1; eBioscience), CD127 PE (hIL-7R-M21; BD), and CD95 Pacific blue (DX2; eBioscience) after gating to each population, which was described above. For the analysis of Bcl6 protein expression, isolated tonsillar Th cells were stained with anti-Bcl6 AlexaFluor 647 (K112-91; BD) after cell permeabilization.

Immunohistochemistry and Immunofluorescence. Tonsils were placed in Tissue-Tek OCT (Sakura), frozen in liquid nitrogen, and stored at −80 °C. For immunohistochemistry, 6-μm cryostat sections were fixed in acetone and air-dried. After treatment with FcReceptor Block and CytoQ Background Buster (Innovex Biosciences) for 20 min each, slides were incubated for 40 min at room temperature with the primary antibody against CD127 (eBioRDR5, purified; eBioscience) diluted in Cyto Q Immuno Diluent. The Stat-Q Peroxidase Staining System with AEC (Innovex Biosciences) was used according to

the manufacturer's protocol. Sections were counterstained with Aqua Hematoxylin (Innovex Biosciences), rinsed in tap water, and prepared on coverslips using Advantage Mounting media from Innovex. Slides images were acquired using a Nikon DXM1200C digital camera and an Olympus BX60 microscope with Plan4x/0.13 objective.

For immunofluorescence, the following antibodies were used: anti-CD4 (RPA-T4, purified; BD) conjugated with Molecular Probes Alexa 568 monoclonal antibody kit, anti-CD127 Alexa647 (eBioRDR5; eBioscience), and anti-CD45RO FITC (UCHL1; eBioscience). Anti-FITC Alexa 488 goat IgG fraction (Molecular Probes) was used as a secondary to enhance the FITC signal. Sections were blocked with both FcReceptor Block and Background Buster, and then, they were incubated for 1 h in the antibodies diluted in CytoQ immunodiluent, rinsed, and incubated in anti-FITC. DAPI (Molecular Probes) was used to stain the nuclei. Slides were mounted with Fluoromount-G (Southern Biotech). They were observed using Metamorph software version 6.2 (Universal Imaging Corporation), a Roper coolsnap HQ camera, and an Olympus BCXCR51 microscope with Plan10x/0.40 objective. Slides were also observed under a Leica SP5 confocal microscope with a 40x/1.25 Planapo objective.

Cell Culture. Sorted Th populations were cocultured with B-cell subsets (2×10^4 cells/well each for 8 d for Ig measurements and 5×10^4 cells/well each for 2 d for cytokine measurements) in RPMI medium 1640 (GIBCO) supplemented with 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma), 1% sodium pyruvate (Sigma), 1% nonessential amino acids (Sigma), 50 μ M β -mercaptoethanol (Sigma), 50 μ g/mL gentamycin (GIBCO), and 10% heat-inactivated FCS (ATCC) in the presence of SEB (1 μ g/mL, Sigma-Aldrich) in U-bottomed 96-well plates. The concentrations of Igs (IgM, IgG, and IgA) were determined by ELISA as described previously (51). Indicated cytokines were measured with Luminex (51). The concentrations of CXCL13 and soluble FAS-L (TNFSF6; both from R&D Systems) were determined by ELISA. Sorted Th populations (5×10^4 cells/well) were also stimulated for 2 d with CD3/CD28 beads (0.2 μ L/well) for cytokine measurement. In blocking experiments, the following reagents were added to the cocultures: anti-ICOS blocking mAb (10 μ g/mL; Ancell), IL-21R/Fc (20 μ g/mL; R&D Systems), anti-Fas blocking antibody (20 μ g/mL; Millipore), and isotype-matched controls. In some experiments, titrated amounts of IL-21 (R&D Systems) were added to the cocultures. For the analysis of plasma cell differentiation, naïve B cells labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes) were cultured for 8 d with Th populations. The cultured cells were stained with anti-CD3 PE-Cy5, anti-CD4 Pacific Blue, and anti-CD38 APC, and the frequency of CFSE⁺CD38^{hi} plasmablast cells within CD3⁺CD4⁺ cells was analyzed by FACS Canto II. In some experiments, Th populations

were cultured with naïve B cells for 5 d in the presence of SEB and then assessed for their expression of ICOS and PD1.

Real-Time PCR. Total RNA was extracted from tonsil Th populations before or after culture with CD3/CD28 mAb-coated beads (5×10^4 cells/0.2 μ L beads per well; Dynal) using the RNeasy Mini kit (QIAGEN) and reverse-transcribed into cDNA in a 96-well plate using the High Capacity cDNA Archive kit (Applied Biosystems). The primer pairs (Integrated DNA Technology) used in this study were designed using the Roche Primer Design Program. Primer sequences were as follows: *BCL6* (accession number NM_001706.2) forward primer: 5'-ttccgtacaaagggaac-3', reverse primer: 5'-tgcaacgatagggtttctca-3'; *PRDM1* (accession number NM_001198.2) forward primer: 5'-gtgggtgggttaatcgggttg-3', reverse primer: 5'-gaagctccctctggaataga-3'; *FASLG* (accession number NM_000639.1) forward primer: 5'-tggggatgtttcagctcttc-3', reverse primer: 5'-tgtgcatctggctggtagac-3'; *IL-21* (accession number NM_021803.2) forward primer: 5'-aggaaaccactctccacaaa-3', reverse primer: 5'-gaatcacatgaaggc-atgtt-3'; *CXCL13* (accession number NM_006419.2) forward primer: 5'-tctctgtctctcatgctgct-3', reverse primer: 5'-tcaagcttggtgaatagacctcca-3'.

Real-time PCR was set up with Roche Probes Master reagents and Universal Probe Library hydrolysis probes. PCR reactions were performed on the LightCycler 480 (Roche Applied Science) followed these conditions: step 1 (denaturation) at 95 °C for 5 min, step 2 (amplification for 45 cycles) at 95 °C for 10 s and 60 °C for 30 s, and step 3 (cooling) at 40 °C for 30 s. The expression of each gene was normalized to that of a housekeeping gene *HRPT1*.

Western Blotting. Total proteins were extracted from sorted tonsil Th populations using radioimmunoprecipitation assay buffer (Sigma-Aldrich) supplemented with 1% protease inhibitor mixture (Sigma-Aldrich). Equal amounts of protein per sample were separated on NuPAGE (Invitrogen) 4–12% Bis-Tris gradient gels and transferred to PVDF membranes (Invitrogen). Membranes were incubated with Abs against Bcl6 (clone D8) and Blimp-1 (clone 6D3) antibody (Santa Cruz Biotechnology, Inc.) followed by HRP-conjugated anti-mouse or anti-rat, respectively (Santa Cruz Biotechnology). Equal protein loading was confirmed using goat antiactin (clone I-19; Santa Cruz Biotechnology, Inc). Densitometry score was determined with ImageJ software.

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