

# CXC Chemokine Receptor 5 Expression Defines Follicular Homing T Cells with B Cell Helper Function

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## Abstract

Leukocyte traffic through secondary lymphoid tissues is finely tuned by chemokines. We have studied the functional properties of a human T cell subset marked by the expression of CXC chemokine receptor 5 (CXCR5). Memory but not naive T cells from tonsils are CXCR5<sup>+</sup> and migrate in response to the B cell-attracting chemokine 1 (BCA-1), which is selectively expressed by reticular cells and blood vessels within B cell follicles. Tonsillar CXCR5<sup>+</sup> T cells do not respond to other chemokines present in secondary lymphoid tissues, including secondary lymphoid tissue chemokine (SLC), EBV-induced molecule 1 ligand chemokine (ELC), and stromal cell-derived factor 1 (SDF-1). The involvement of tonsillar CXCR5<sup>+</sup> T cells in humoral immune responses is suggested by their localization in the mantle and light zone germinal centers of B cell follicles and by the concomitant expression of activation and costimulatory markers, including CD69, HLA-DR, and inducible costimulator (ICOS). Peripheral blood CXCR5<sup>+</sup> T cells also belong to the CD4<sup>+</sup> memory T cell subset but, in contrast to tonsillar cells, are in a resting state and migrate weakly to chemokines. CXCR5<sup>+</sup> T cells are very inefficient in the production of cytokines but potently induce antibody production during coculture with B cells. These properties portray CXCR5<sup>+</sup> T cells as a distinct memory T cell subset with B cell helper function, designated here as follicular B helper T cells (T<sub>FH</sub>).

Key words: chemokines • CXC chemokine receptor 5 • lymphocytes • B cell follicle • antibodies

## Introduction

T cell-dependent immune responses to pathogens are initiated in specialized lymphoid tissues, including LNs, Peyer's patches (PPs),<sup>1</sup> and the spleen. The outcome of these defensive mechanisms relies on the finely tuned traffic of T and B cells as well as antigen-presenting cells, suggesting that chemokines may be involved in the recruitment and proper positioning of leukocytes within these compartments. Chemokines comprise a large family of structurally related chemoattractant proteins that regulate the composi-

tion of cellular infiltrates at sites of inflammation or, alternatively, the physiological leukocyte migration during hematopoiesis, antigen sampling in secondary lymphoid tissues, and immune surveillance (1–6). This report focuses on a particular subset of T cells with homing selectivity for B cell follicles in secondary lymphoid tissues.

Chemokines shown to be produced in LNs, PPs, or the spleen include secondary lymphoid tissue chemokine (SLC; CCL21) (systematic nomenclature for human chemokines available at <http://cytokine.medic.kumamoto-u.ac.jp/>), EBV-induced molecule 1 ligand chemokine (ELC; CCL19), B cell-attracting chemokine 1 (BCA-1; CXCL14), stromal cell-derived factor 1 (SDF-1; CXCL12), and monocyte-derived chemokine (MDC; CCL22 [6–8]). In addition, several typical inflammatory chemokines are upregulated in inflamed rather than resting LNs, which may reflect an enhanced T cell activation status. In contrast to SDF-1 and MDC, the role of SLC and ELC in controlling cellular traffic in secondary lymphoid tissues is well es-

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<sup>1</sup>Abbreviations used in this paper: BCA-1, B cell-attracting chemokine 1; CCR7, CC chemokine receptor 7; CXCR5, CXC chemokine receptor 5; DIG, digoxigenin; ELC, EBV-induced molecule 1 ligand chemokine; HEV, high endothelial venule; HPF, high power field; ICOS, inducible costimulator; PP, Peyer's patch; SDF-1, stromal cell-derived factor 1; SLC, secondary lymphoid tissue chemokine; TBS, Tris-buffered saline.

established. The two chemokines are produced within T zone areas but are absent in B cell follicles, and their expression is dependent on TNF and/or lymphotoxin (LT) $\alpha/\beta$  (4, 6, 7). CC chemokine receptor 7 (CCR7), the selective receptor for SLC and ELC, is present on the bulk of resting T cells in peripheral blood and mature dendritic cells and mediates rapid adhesion to integrin ligands and chemotactic migration (1–4, 6, 7). Importantly, mutant mice with defective SLC/ELC production (9–12) and gene-targeted mice that lack CCR7 (13) show severe defects in secondary lymphoid tissue architecture, notably in the proper positioning of T and B cells and in the homing function of mature dendritic cells. Consequently, these mutant mice display markedly delayed kinetics in T cell-dependent immune responses whereas antibody responses remain largely unaffected.

What about the involvement of chemokines in the regulation of humoral responses? BCA-1 is a highly efficient chemoattractant for mature B cells that uniformly express its receptor CXC chemokine receptor 5 (CXCR5 [14–16]). In mice, the lack of CXCR5 or BLC (the murine homologue of human BCA-1) resulted in abnormal lymphoid tissue formation (17, 18), notably follicular organization in the spleen and PPs, whereas transgenic mice expressing BLC in pancreatic islets showed secondary lymphoid tissue-like structures at these sites (19), suggesting an important role for BCA-1 in the organization of human B cell follicles. However, CXCR5 deficiency does not prevent murine B cell responses, and occasionally small germinal center-like B cell aggregates are observed (20). In mice, BCA-1 transcripts are detected in follicles and, similar to SLC and ELC, its expression is also dependent on TNF and/or LT $\alpha/\beta$  (11, 15, 21, 22). In humans, BCA-1 expression was mainly studied in *Helicobacter pylori*-associated lymphoid tissue and gastric lymphomas (23). In addition to B cells, low numbers of human peripheral blood T cells and a larger fraction of tonsillar T cells express CXCR5 but their function is not known (17, 24). Here we report the migration and effector properties of human CXCR5<sup>+</sup> T cells present in blood and tonsils. Distinct migration properties and the ability to induce antibody production during coculture with B cells indicate that CXCR5 expression marks a T cell fraction with unique effector function.

## Materials and Methods

**Antibodies.** Antibodies to human proteins were purchased from the following sources: rat antibodies to IL-5 (TRFK5) and IL-10 (JES3-9D7), and mouse antibodies to CD4 (RPA-T4), CD40 (5C3), CD45RO (UCHL1), CD45RA (HI100), CD62L (DREG-56), CD69 (FN50), CD134 (ACT35), CD152 (BNI3), and CD154 (TRAP-1) were all from BD PharMingen; mouse antibodies to CCR6 (53103.111), CXCR3 (49801.111), CXCR4 (12G5), and goat IgG to BCA-1 (AF470) were from R&D Systems; mouse antibodies to IFN- $\gamma$  (25723.11), IL-2 (5344.111), IL-4 (3010.211), and IL-13 (JES10-5A2) were from Becton Dickinson; mouse antibody to CD71 (DF1513) was from

Sigma-Aldrich; mouse antibodies to CD4 (MT310), CD19 (HD37), CD20 (L26), CD21 (1F8), CD25 (ACT-1), and CD31 (JC/70A), rabbit IgG to CD3 (A0452), and bovine S-100 were from Dako; mouse antibodies to CCR3 (7B11) and CCR5 (5C7) were from LeukoSite, Inc.; and mouse antibody to HLA-DR (L243) was from Research Diagnostics Inc. Secondary and control antibody reagents were from the following sources: goat anti-rabbit IgG (F-1262), mouse IgG1, and goat IgG were from Sigma-Aldrich; biotinylated rabbit anti-rat IgG (E0468) and mouse IgG2a were from Dako; goat anti-mouse MicroBead IgG (484-01) and goat anti-rabbit MicroBead IgG (486-02) were from Miltenyi Biotec; mouse IgG2b was from R&D Systems; rabbit IgG was from Zymed Laboratories; rat IgG was from BD PharMingen; biotinylated donkey anti-goat/sheep Ig (AB360) was from The Binding Site. The following antibodies were from noncommercial sources: rat antibody to CCR7 (3D12; M. Lipp, Max-Delbrück Center for Molecular Medicine, Berlin, Germany); mouse antibody to inducible costimulator (ICOS; F44; R.A. Kroczeck, Robert Koch Institute, Berlin, Germany); mouse antibody to CD3 (Tr66; A. Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland); and rabbit IgG to CXCR5 (23).

**Cell Preparation and Cultures.** Human PBLs were isolated from donor blood buffy coats by centrifugation on Ficoll-Paque followed by Percoll (25). For the measurement of intracellular cytokines, CD4<sup>+</sup>CD45RO<sup>+</sup> PBLs were negatively selected by removing CD8<sup>+</sup>, CD14<sup>+</sup>, CD16<sup>+</sup>, CD45RA<sup>+</sup>, and CD56<sup>+</sup> cells using the magnetic cell sorting system from Miltenyi Biotec. The cells obtained were also used to study the induction of CXCR5 expression after removal of the CXCR5<sup>+</sup> cells. Tonsils from individuals undergoing tonsillectomy were mechanically dispersed and lymphocytes were isolated by centrifugation on Ficoll-Paque. CD4<sup>+</sup>CD45RO<sup>+</sup>CXCR5<sup>+</sup> tonsillar cells were isolated by first removing CD8<sup>+</sup>, CD20<sup>+</sup>, CD45RA<sup>+</sup>, and CD56<sup>+</sup> cells and then by positive selection of CXCR5<sup>+</sup> cells (>97% purity). Lymphocytes were cultured in RPMI 1640 containing 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 50  $\mu$ g/ml penicillin/streptomycin,  $5 \times 10^{-5}$  M 2-mercaptoethanol (GIBCO BRL), and 10% heat-inactivated FCS (Inotech/Biological Industries). Cells were stimulated with 5  $\mu$ g/ml anti-CD3 precoated on tissue plates or 1  $\mu$ g/ml PHA (Wellcome) in the presence of 20 U/ml IL-2.

**T Cell Effector Functions.** For detection of intracellular cytokines, purified CD4<sup>+</sup>CD45RO<sup>+</sup> PBLs were stimulated with 10 ng/ml PMA and 1  $\mu$ g/ml ionomycin for 6 h, and 10  $\mu$ g/ml Brefeldin A was added for the last 4 h. Cells were then fixed with 2% paraformaldehyde, permeabilized with 0.2% saponin/2% FCS in PBS, and stained with the appropriate antibodies to cytokines for flow cytometric analysis (FACScan<sup>TM</sup>; Becton Dickinson). Labeling of surface proteins was performed before fixation.

T cell help in antibody production was studied as follows. Tonsillar T cells (CD4<sup>+</sup>CXCR5<sup>+</sup> and CD4<sup>+</sup>CXCR5<sup>-</sup>) and B cells (CD4<sup>-</sup>CD8<sup>-</sup>CXCR5<sup>+</sup>) were sorted by FACS<sup>®</sup> and cocultured in 96-well plates at  $10^5$  cells/well each of T and B cells in the absence of IL-2 for 10 d. IgM, IgG, and IgA contents in the culture supernatants were determined by ELISA.

**T Cell Chemotaxis.** Cell migration was measured in 48-well chemotaxis chambers (Neuro Probe Inc. [25]). In brief, chemokines in HEPES-buffered RPMI 1640 supplemented with 1% pasteurized plasma protein (Swiss Red Cross Laboratory) were added to the lower wells and 100,000 cells in the same medium to the upper wells. Polyvinylpyrrolidone-free polycarbonate membranes (Poretics Corp.) with 3- $\mu$ m pores coated with type

IV collagen were used. After incubation for 1.5 h, the membrane was removed, washed on the upper side with PBS, fixed, and stained. Migrated cells were counted microscopically at 1,000 $\times$  magnification in five randomly selected fields per well. The assay was performed in triplicates.

**Northern Blot Analysis.** Total RNA was extracted from CXCR5<sup>+</sup> T cells immediately after isolation or after stimulation with anti-CD3, and 10- $\mu$ g samples were analyzed by Northern blotting using <sup>32</sup>P-labeled receptor DNA fragments (10<sup>9</sup> cpm/mg DNA) as hybridization probes (26). The following probes were used: a 550-bp BamH1 fragment for CXCR4 (27), a 550-bp BstX1 fragment for CXCR5 (14), and a 430-bp BstX1 fragment for CCR7 (26).

**Immunohistochemistry and In Situ Analysis.** Tonsillar tissue sections were either snap frozen in liquid nitrogen or, alternatively, formaldehyde fixed and paraffin embedded. For immunostaining, sections of frozen tissue were fixed with acetone, air dried, and incubated in 10% rabbit or swine serum in Tris-buffered saline (TBS) containing 0.01% Tween 20, and then further processed as the paraffin-embedded sections. Optimal staining for S-100, CD21, CD45RO, and HLA-DR required incubation of paraffin-embedded sections with proteinase (proteinase XXV; Sigma-Aldrich) for 6 min at 37°C, and identification of CD3<sup>+</sup> or CD20<sup>+</sup> cells was improved by three times boiling of paraffin-embedded sections (microwave, 5 min each time) in 10 mM citrate buffer, pH 6.0, before incubation with primary antibodies in TBS containing 1% rabbit or swine serum plus either 2% Tween 20 (S-100) or 0.01% Tween 20 (others). After overnight incubation, sections were treated by standard alkaline phosphatase anti-alkaline phosphatase (APAAP) or avidin-biotin complex (ABC) techniques (Dako) for mouse or rabbit/goat antibodies, respectively, stained with New Fuchsin solution (New Fuchsin Kit; Dako), counterstained with Mayer's hematoxylin (Fluka), and mounted using Aquamount (BDH Laboratory Supplies). For double immunofluorescence staining, frozen sections were incubated with goat anti-BCA-1 antibody and mouse anti-CD31 antibody as described above, then further processed in TBS/0.5% BSA with biotinylated sheep anti-goat antibody/streptavidin-Cy5-RPE (C0050; Dako) for BCA-1, followed by FITC-goat anti-mouse Ig (F0479; Dako) for CD31, and, finally, mounted with Mowiol (Aventis) plus 0.02% diazabicyclo[2.2.2]octane (DABCO; Fluka) to avoid bleaching. Stained sections were analyzed by light or confocal microscopy (Nikon microscope Eclipse E600 or ZEISS Laser Scanning Microscope LSM410).

For in situ hybridization, digoxigenin (DIG)-labeled RNA probes of 368 nucleotides corresponding to the 5' region in the BCA-1 DNA (14) were prepared by in vitro transcription using the DIG RNA labeling kit (Roche Diagnostics). Dewaxed/rehydrated and proteinase-treated paraffin-embedded sections were fixed in 4% paraformaldehyde/PBS and acetylated for 10 min with 26.4 mM acetic anhydride in 0.1 M triethanolamine. After prehybridization in 4 $\times$  SSC, 50% deionized formamide, 2 $\times$  Denhardt's solution, 250  $\mu$ g/ml yeast RNA, and 100  $\mu$ g/ml salmon sperm DNA for 2 h at 50°C, sections were incubated at 50°C overnight with 10 ng of DIG-labeled sense or antisense RNA probes in 4 $\times$  SSC, 1% dextran sulfate, 50% deionized formamide, 2 $\times$  Denhardt's solution, 500  $\mu$ g/ml yeast RNA, and 500  $\mu$ g/ml salmon sperm DNA. After one washing with 2 $\times$  SSC at room temperature for 30 min, RNase T1 (Roche Diagnostics) treatment at 100,000 U/ml, and additional washings at 54°C with 2 $\times$  SSC followed by 0.2 $\times$  SSC, DIG-labeled RNA was detected by anti-DIG antibody coupled to alkaline phosphatase and color development according to the supplier (Roche Diagnostics).

## Results

**CXCR5<sup>+</sup> T Cells in Blood and Tonsils.** Coexpression of cell surface markers with CXCR5 on human T cells was analyzed by flow cytometry (Table I). In peripheral blood, CXCR5 is present on a subpopulation of memory (CD45RO<sup>+</sup>) T cells, but absent on naive (CD45RA<sup>+</sup>) T cells, as reported previously (17, 24). Over 90% of CXCR5<sup>+</sup> T cells express CD4 and <10% express CD8. About 20% of total CD4<sup>+</sup>CD45RO<sup>+</sup> T cells are CXCR5<sup>+</sup>, showing that CXCR5 is less frequent on these cells than on B cells, which uniformly express CXCR5 (16, 17, 24, 28). Circulating CXCR5<sup>+</sup> T cells are in a resting state (CD25<sup>-</sup>, CD69<sup>-</sup>, CD71<sup>-</sup>, HLA-DR<sup>-</sup>) and lack the expression of costimulatory molecules (CD40L, OX40, ICOS), suggesting that they are not involved in ongoing immune responses. In contrast, CXCR5<sup>+</sup> T cells are highly enriched in inflamed tonsillar tissue. Almost all memory T cells (>95%) are CD4<sup>+</sup> and stain positive for CXCR5, whereas naive T cells do not express CXCR5. Furthermore, several activation markers (CD69, HLA-DR, ICOS) are found on the majority of CXCR5<sup>+</sup> T cells, suggesting that this fraction is engaged in tonsillar immune responses (Table I). Other inducible T cell markers, including alternative costimulatory molecules (CD40L, OX40, CTLA-4) or activation/proliferation markers (CD25, CD71), were not detected.

**Modulation of CXCR5 Expression and Migration to BCA-1.** The migration capacity of leukocytes and, consequently, their potential involvement in immune responses, depends largely on the repertoire of chemokine receptors that are functionally expressed. CCR7, which defines homing of circulating T cells to T cell compartments within secondary

**Table I.** Phenotypic Markers on CXCR5<sup>+</sup> T Cells in Blood and Tonsils

Marker	Blood	Tonsils
CD4	95 $\pm$ 2.1 (8)*	92 $\pm$ 4.3 (7)
CD8	5 $\pm$ 1.5 (8)	8 $\pm$ 2.9 (7)
CD45RO	98 $\pm$ 1.9 (8)	97 $\pm$ 2.5 (7)
CD25	1 $\pm$ 0.5 (5)	1 $\pm$ 0.8 (5)
CD69	3 $\pm$ 0.8 (5)	88 $\pm$ 4.1 (5)
CD71	1 $\pm$ 0.5 (5)	2 $\pm$ 1.2 (5)
HLA-DR	2 $\pm$ 0.9 (5)	78 $\pm$ 9.8 (5)
OX40 (CD134)	1 $\pm$ 0.6 (4)	3 $\pm$ 1.4 (4)
CD40L (CD154)	2 $\pm$ 1.9 (4)	2 $\pm$ 1.4 (4)
CTLA-4 (CD152)	1 $\pm$ 0.6 (4)	2 $\pm$ 0.8 (4)
ICOS	4 $\pm$ 1.2 (5)	65 $\pm$ 8.5 (5)
CD62L	82 $\pm$ 4.8 (5)	42 $\pm$ 5.8 (5)

Freshly isolated CXCR5<sup>+</sup>CD3<sup>+</sup> T cells from blood or tonsils were analyzed by three-color flow cytometry.

\*Percentage  $\pm$  SEM of CXCR5<sup>+</sup>CD3<sup>+</sup> T cells expressing the indicated cell surface proteins (gates set at 99% control antibody staining); numbers in parentheses refer to independent cell preparations analyzed.

**Table II.** Chemokine Receptor Profile on CXCR5<sup>+</sup>CD4<sup>+</sup> T Cells in Blood and Tonsils

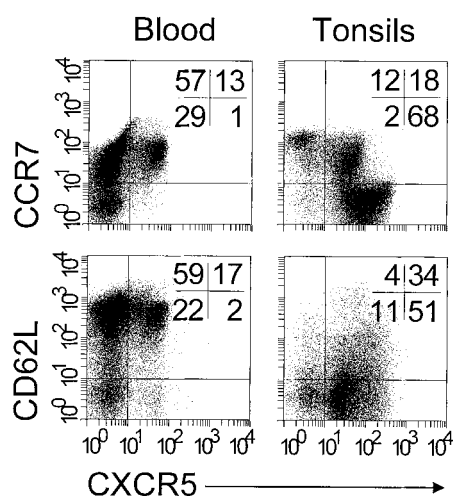
Receptor	Blood	Tonsils
CXCR3	25 ± 3.2 (5)*	30 ± 4.3 (5)
CXCR4 <sup>‡</sup>	34 ± 15 (5)	29 ± 12 (5)
CCR3	3 ± 2.9 (3)	9 ± 5.0 (3)
CCR5	4 ± 3.2 (3)	14 ± 9.4 (3)
CCR6	32 ± 5.5 (4)	36 ± 6.8 (4)
CCR7	97 ± 2.5 (5)	28 ± 6.2 (5)

Freshly isolated CXCR5<sup>+</sup>CD4<sup>+</sup> T cells from blood or tonsils were analyzed by three-color flow cytometry for coexpression of chemokine receptors.

\*Percentage ± SEM of CXCR5<sup>+</sup>CD4<sup>+</sup> T cells expressing the indicated chemokine receptors (gates set at 99% control antibody staining); numbers in parentheses refer to independent cell preparations analyzed.

<sup>‡</sup>All blood and tonsillar T cells express high levels of CXCR4 after overnight culture under nonstimulatory conditions (see also Fig. 2 c).

lymphoid tissues (29–31), is coexpressed on virtually all circulating CXCR5<sup>+</sup> T cells (Table II). This is in striking contrast to the marked reduction of CCR7 expression on tonsillar CXCR5<sup>+</sup> T cells (Fig. 1), suggesting that SLC and ELC, the ligands of CCR7, may have caused its downmodulation (2). Similarly, L-selectin (CD62L) expression is reduced on tonsillar CXCR5<sup>+</sup> T cells compared with blood CXCR5<sup>+</sup> T cells, implying that the majority of tonsillar CXCR5<sup>+</sup> T cells have recently immigrated from circulation. No striking differences between blood and tonsil-



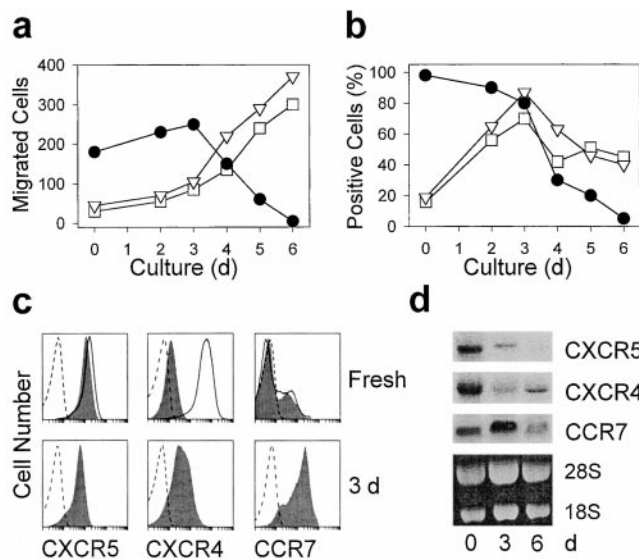
**Figure 1.** CCR7 and CD62L are downmodulated on tonsillar CXCR5<sup>+</sup> T lymphocytes. Freshly isolated blood and tonsillar lymphocytes were triple stained with antibodies to CD4, CXCR5, and CCR7 or CD62L and analyzed by flow cytometry. Dot plots show cells gated for the expression of CD4. Quadrants were set according to the staining of control antibodies and the percentage of cells in each quadrant is indicated. Comparable results were obtained with five additional blood and tonsillar cell preparations.

lar CXCR5<sup>+</sup> T cells were observed for other chemokine receptors. CXCR4 is unique in its uniform but weak expression on freshly isolated blood and tonsillar T cells (see below). Fractions of similar size stain positive for CXCR3, CCR3, CCR5, and CCR6, whereas other chemokine receptors were not detected.

Freshly isolated tonsillar CXCR5<sup>+</sup> T cells migrated readily in response to BCA-1, whereas responsiveness to ELC and SDF-1 was routinely low or undetectable (Fig. 2 a). Responsiveness to BCA-1 was moderately enhanced when the cells were treated with anti-CD3 or PHA (not shown). Prolonged activation resulted in complete loss of chemotaxis and receptor expression, and this effect correlates with the appearance of T cell blasts (Fig. 2, a and b). CXCR5 transcripts were readily detected in freshly isolated CXCR5<sup>+</sup> T cells and decreased to undetectable levels by day 6 of culture (Fig. 2 d). These observations are in striking contrast to changes in ELC- and SDF-1-mediated responses and in the expression of their receptors. Initially, chemotaxis to ELC and SDF-1 remained low until responsiveness to BCA-1 started to decline, i.e., T cell blast and cell proliferation became evident, and then rapidly increased to levels exceeding the maximal responses observed with BCA-1 (Fig. 2 a). CCR7 protein and mRNA expression increased during the first 3 d of activation, followed by a slow decline. Of note, these changes do not correlate with changes in T cell migration, as marked responses were observed only in the phase of chemokine receptor downmodulation (Fig. 2, a and b). CXCR4 is uniformly but at low levels present on freshly isolated tonsillar CXCR5<sup>+</sup> T cells and high level expression is restored by overnight culture under nonstimulatory conditions (Fig. 2 c), suggesting receptor relocation from intracellular stores rather than de novo gene expression (32–34). In this respect, CXCR4 expression clearly differs from the expression of CXCR5 and CCR7, which was not affected by overnight culture (Fig. 2 c). However, similar to ELC/CCR7, enhancement of chemotaxis to SDF-1 occurred late in culture and was not related to changes in cell surface CXCR4.

Peripheral blood CXCR5<sup>+</sup> T cells migrated poorly to BCA-1 (25 ± 5 migrated cells/5 high power fields [HPFs]; *n* = 3). Similar to tonsillar CXCR5<sup>+</sup> T cells, activation with anti-CD3 or PHA led to a transient enhancement of migration, reaching maximal levels by day 3 (92 ± 6 migrated cells/5 HPFs; *n* = 3), followed by a rapid decline in both BCA-1-mediated migration and CXCR5 expression. CXCR5 expression in naive T cells was not achieved by common stimulation protocols (anti-CD3 with or without anti-CD28) and, thus, may require accessory stimulation provided by antigen-presenting cells. Loss of CXCR5 expression and responsiveness to BCA-1 was routinely observed during in vitro culture of T cells, as evidenced by 10 independent cell lines established from sorted CXCR5<sup>+</sup> T cells from blood or tonsils. Also, CXCR5 expression was completely absent in numerous unrelated T cell lines with defined antigen specificity and cytokine production profile.

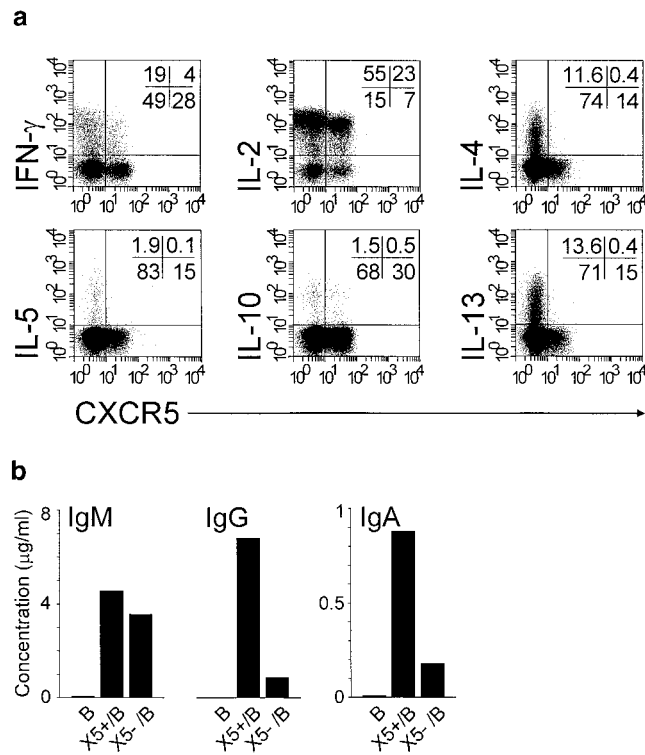
**Cytokine Profile of CXCR5<sup>+</sup> T Cells.** The pattern of cytokine production was studied by flow cytometry in



**Figure 2.** Inhibition of migration to BCA-1 and CXCR5 expression during culture of tonsillar CXCR5<sup>+</sup> T cells. Freshly isolated tonsillar CXCR5<sup>+</sup> T cells were activated with immobilized anti-CD3 for up to 6 d and examined for *in vitro* migration (a); chemokine receptor expression was assessed by flow cytometry (b and c) and Northern blot analysis (d). (a) Chemotaxis in response to 1,000 nM BCA-1 (●), 100 nM SDF-1 (□), and 100 nM ELC (∇); mean number of migrated cells per 5 HPFs in triplicate wells). The data are representative of five independent experiments. (b) The percentage of cells positive for CXCR5 (●), CXCR4 (□), and CCR7 (∇), with gates set at 99% isotype-matched control antibody staining. (c) Expression of CXCR5, CXCR4, and CCR7 (shaded) in freshly isolated cells and in cells treated with anti-CD3 for 3 d. Broken lines represent the staining of isotype-matched control antibodies, and solid lines show chemokine receptor expression after overnight culture under nonstimulatory conditions. (d) Northern blot analysis of CXCR5, CXCR4, and CCR7 transcripts with total RNA from freshly isolated cells and after treatment with anti-CD3 for 3 and 6 d.

CXCR5<sup>+</sup> and CXCR5<sup>-</sup> peripheral blood T cells after potent polyclonal stimulation (35). Only minor populations within CXCR5<sup>+</sup> T cells were capable of producing IFN- $\gamma$  ( $11.2 \pm 1.5\%$ ; mean  $\pm$  SEM); IL-4, IL-5, IL-10, and IL-13 were not produced ( $<3\%$ ; Fig. 3 a). As expected, both CXCR5<sup>+</sup> and CXCR5<sup>-</sup> T cells synthesized IL-2 equally well ( $77.2 \pm 1.0$  and  $80.0 \pm 1.1\%$ , mean  $\pm$  SEM, respectively). In contrast, CXCR5<sup>-</sup> T cells produced more prominently IFN- $\gamma$  ( $28.4 \pm 0.6\%$ , mean  $\pm$  SEM), IL-4 ( $12.0 \pm 1.5\%$ , mean  $\pm$  SEM), and IL-13 ( $13.5 \pm 2.6\%$ , mean  $\pm$  SEM; Fig. 3 a). Of note, the loss of CXCR5 expression in long-term cultures was accompanied by the acquisition of enhanced cytokine production, as shown in cell lines generated from sorted CXCR5<sup>+</sup> T cells. Except for IL-2 and IFN- $\gamma$ , CXCR5<sup>+</sup> T cells from tonsils did not produce cytokines, which fully agrees with the results obtained with blood CXCR5<sup>+</sup> T cells.

**B Cell Helper Function of CXCR5<sup>+</sup> T Cells.** Coculture experiments with tonsillar T and B cells showed that CXCR5<sup>+</sup> T cells are potent inducers of antibody production (Fig. 3 b). CXCR5<sup>+</sup> T cells enhanced the production of IgG and IgA by a factor of  $6.3 \pm 2.1$  (mean  $\pm$  SEM,  $n = 4$ ) and  $4.6 \pm 1.7$  (mean  $\pm$  SEM,  $n = 4$ ), respectively,



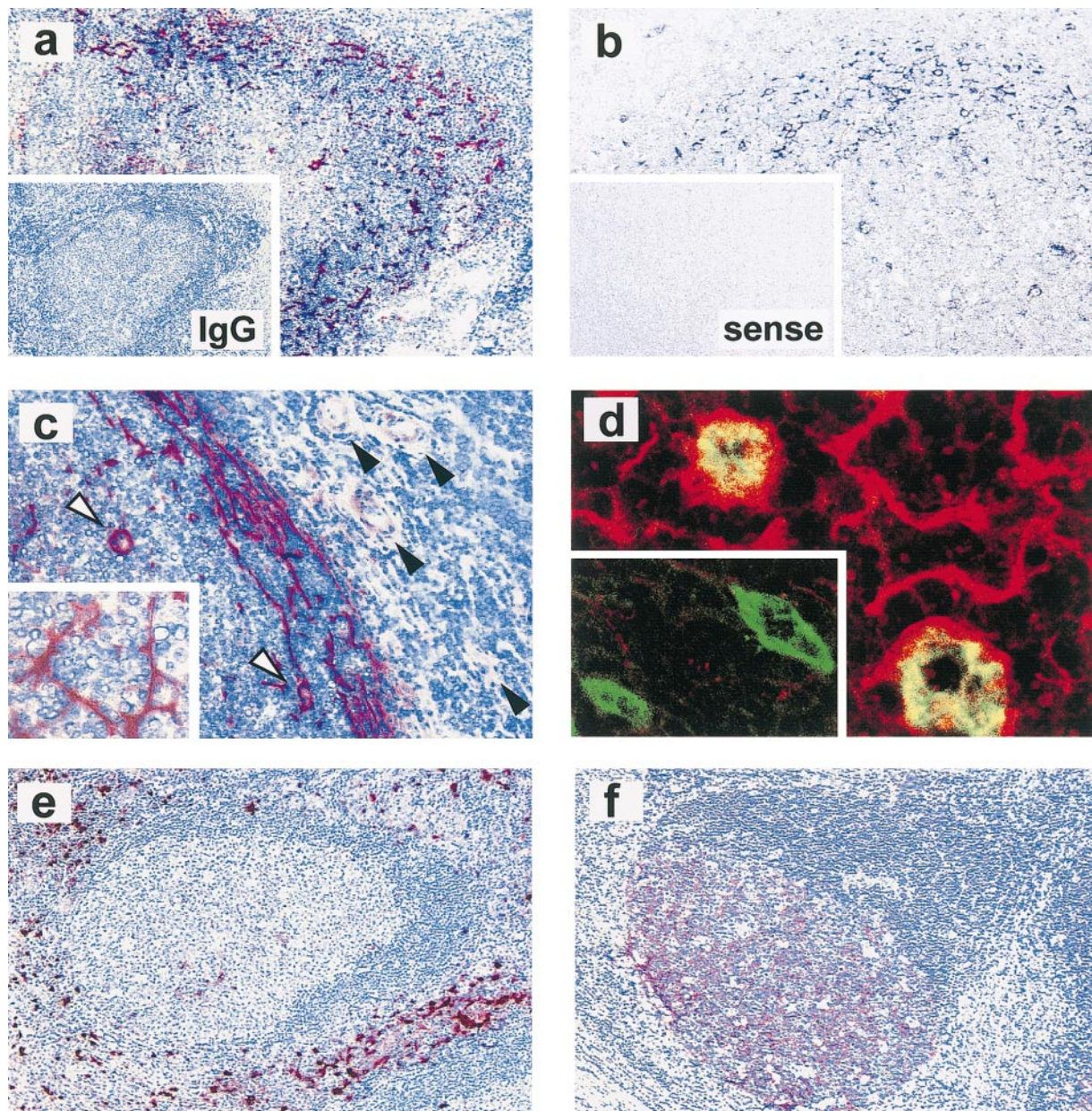
**Figure 3.** Lack of cytokine production but induction of IgG/IgA synthesis by CXCR5<sup>+</sup> T cells. (a) CD4<sup>+</sup>CD45RO<sup>+</sup> T cells were isolated from blood by negative selection. After polyclonal stimulation, CXCR5<sup>+</sup> and CXCR5<sup>-</sup> CD4<sup>+</sup> T cells were examined for intracellular production of IFN- $\gamma$ , IL-2, IL-4, IL-5, IL-10, and IL-13 by three-color flow cytometry. Dot plots show cells gated for CD4. Quadrants were set according to the staining of isotype-matched control antibodies and the percentage of cells in each quadrant is indicated. Results are representative of a total of 11 experiments with memory T cell preparations from different blood and tonsil donors. (b) CXCR5<sup>+</sup> T cells induce antibody production in tonsillar B cells. Tonsillar B cells were cultured for 10 d either alone (B) or in the presence of CXCR5<sup>+</sup> (X5<sup>+</sup>/B) or CXCR5<sup>-</sup> (X5<sup>-</sup>/B), and IgG, IgA, and IgM concentrations in the culture supernatants were determined by ELISA. A representative example out of four independent experiments is shown.

compared with CXCR5<sup>-</sup> T cells. Both T cell fractions were equally potent in induction of IgM secretion ( $1.3 \pm 0.6$ -fold [mean  $\pm$  SEM,  $n = 4$ ] enhancement by CXCR5<sup>+</sup> T cells), and IgM levels were consistently lower than those of IgG, suggesting that CXCR5<sup>+</sup> T cells affected B cell Ig switching (IgM to IgG/IgA) and/or memory B cell activation. B cells cultured in the absence of T cells produced marginal levels of antibodies ( $<60$  ng/ml for IgG and IgM, and  $<30$  ng/ml for IgA). In contrast to tonsillar T cells, CXCR5<sup>+</sup> T cells from peripheral blood did not support antibody production in B cell cultures in the absence of exogenous stimuli, which correlates with their resting state.

**Colocalization of CXCR5<sup>+</sup> T Cells and BCA-1 Production in Tonsils.** Prominent attraction by BCA-1 and reduced responsiveness to SLC, ELC, and SDF-1 suggest that tonsillar CXCR5<sup>+</sup> T cells are recruited to sites of BCA-1 production. Immunohistochemistry and *in situ* hybridization of tonsillar tissue sections revealed the highly selective ex-

pression of BCA-1 in the follicular mantle zone and, albeit less prominently, in the light zone of germinal centers (Fig. 4, a and b), which markedly contrasts with the T zone-selective expression of SLC and ELC (4, 6, 7). Positivity in the mantle zone is associated with reticular cells, as demonstrated by the intense staining of dendrite-like cellular extensions (Fig. 4 c, inset). BCA-1-producing cells may represent a distinct class of dendritic cells, as their localization differs from follicular (CD21<sup>+</sup>) or interdigitating (S-100<sup>+</sup>)

dendritic cells (Fig. 4, e and f). In addition, prominent BCA-1 production is found on high endothelial venules (HEVs) within follicles but not in the T zone, as shown by single staining (Fig. 4 c, arrowheads). This is supported by confocal microscopy with fluorescence-labeled antibodies to BCA-1 (red) and CD31 (green), demonstrating colocalization in follicular HEVs (yellow) (Fig. 4 d; inset shows T zone HEVs). In 20 randomly selected areas in tonsillar sections (0.32 mm<sup>2</sup>/each), a total of 699 vessels were identi-



**Figure 4.** BCA-1 expression in tonsillar follicles. (a and c) Immunostaining of BCA-1 (red) in frozen tonsillar sections; inset in panel a shows control staining with isotype-matched control. (b) In situ hybridization of BCA-1 transcripts using an antisense probe (black) on paraffin sections; no staining is observed with a sense probe (inset in b). Positivity in the mantle zone is associated with reticular cells, as highlighted in the inset of c, and follicular HEVs (white arrowheads in c) but not T zone HEVs (black arrowheads in c). (d) Confocal microscopic analysis of BCA-1 (red) and CD31 (green) expression in follicular HEVs and T zone HEVs (inset in d). Immunostaining of interdigitating dendritic cells with an anti-S-100 antibody (e) and follicular dendritic cells with an anti-CD21 antibody (f) is shown to highlight the nonoverlapping localization of BCA-1-producing reticular cells. Original magnifications: (a, e, and f)  $\times 100$ ; (b and c)  $\times 200$ ; and (d)  $\times 1,600$ .

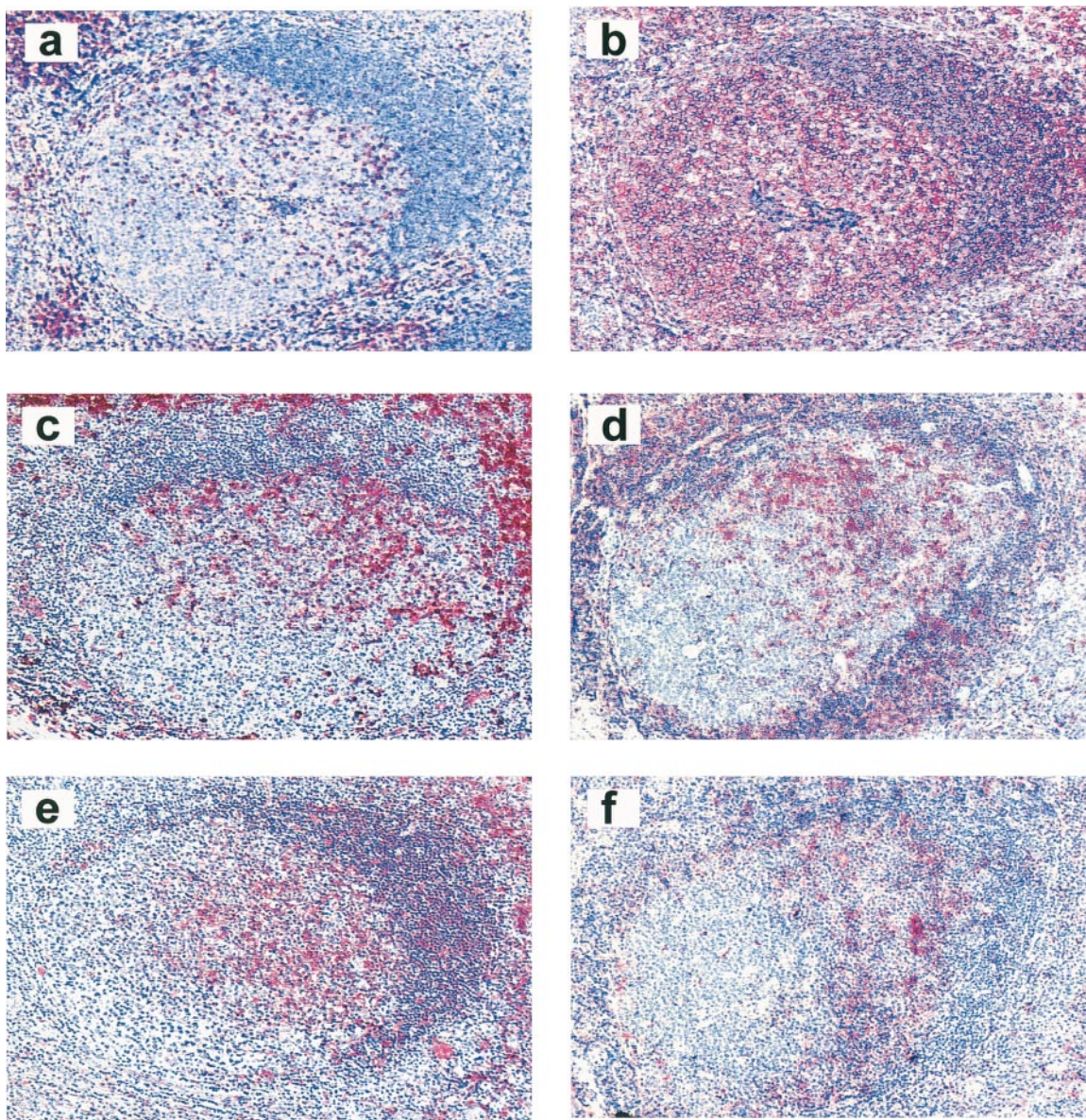
fied by anti-CD31 staining and 99 out of 101 BCA-1<sup>+</sup> vessels were located in the follicular mantle. This selectivity in vascular BCA-1 expression suggests that circulating CXCR5<sup>+</sup> cells could be directly recruited into B cell follicles via BCA-1–induced attachment to follicular HEVs.

Numerous CD3<sup>+</sup> T cells are present in the light zone of germinal centers, and similar high numbers of cells at this location stained positive for CD4 (not shown), HLA-DR, CD69, or ICOS (Fig. 5). These data agree with the follicular localization of tonsillar CXCR5<sup>+</sup> T cells, which express these activation markers (Table I). Follicular T cells are greatly outnumbered by B cells, which all express CXCR5 (not shown [16, 17, 24, 28]). Follicular mantle zones are areas of intensive cellular interactions, and dense nuclear staining with eosin/hematoxylin greatly diminishes over-

lapping signals obtained by immunohistochemistry. Nevertheless, positive staining for CD3, CD4, CD45RO, HLA-DR, CD69, and ICOS was clearly detected and, as expected, was much less prominent than staining for CD20 (B cells).

## Discussion

Recruitment of lymphocytes into secondary lymphoid tissue requires the presence of CD62L, which mediates initial adhesive interactions of lymphocytes on HEVs (36), and CCR7, which triggers subsequent firm adhesion through integrin activation (30, 31, 37–39). This mechanism is operative for naive T cells, whose numbers are drastically reduced in secondary lymphoid tissues of CCR7- or



**Figure 5.** Phenotypic characterization of follicular T cells. Immunostaining (red) on paraffin sections is shown for CD3 (a), CD20 (b), CD45RO (c), and HLA-DR (e), and on frozen sections for CD69 (d) and ICOS (f). Original magnification:  $\times 100$ .

SLC-deficient mice (10, 13), and may also apply to memory T cells, which give rise to effector cells during recognition of recall antigens. The majority (65–95%;  $n = 5$ ) of resting/memory T cells in peripheral blood are double positive for CD62L and CCR7, and termed central memory T cells ( $T_{CM}$ ) to designate antigen-experienced T cells with secondary lymphoid tissue-homing characteristics (29). CXCR5 is present on a subset of circulating  $CD4^+$  memory T cells with poor cytokine production capabilities that coexpress CD62L and CCR7. CXCR5 expression is negligible (3%) on  $CD8^+$  T cells and is absent on naive T cells. Accordingly, peripheral CXCR5<sup>+</sup> T cells represent a subpopulation of  $T_{CM}$  and, thus, differ from CCR7<sup>-</sup> effector memory T cells ( $T_{EM}$ ), which are proposed to directly participate in immune responses at inflammatory sites (29).

In addition to CCR7, CXCR5<sup>+</sup> T cells uniformly express low levels of CXCR4, but there is no evidence for a role of this receptor in T cell traffic through secondary lymphoid tissues. Except for CXCR3, CXCR5<sup>+</sup> T cells lack chemokine receptors that are typically involved in recruitment of effector/memory T cells to inflammatory sites in peripheral tissues. This chemokine receptor profile suggests that CXCR5<sup>+</sup> T cells home preferentially into secondary lymphoid tissues and, therefore, T cells from tonsils were selected for further studies. Tonsils of patients with pharyngeal inflammation contain a high density of secondary follicles separated by small T zone areas, indicative of intensive B cell activation processes. Therefore, CXCR5<sup>+</sup> T cells within this tissue may actively participate in local immune responses. Practically all (>95%)  $CD4^+$  memory T cells in tonsils are CXCR5<sup>+</sup> and the majority express activation markers (CD69, HLA-DR, ICOS) suggesting their engagement in B cell activation. Drastic reduction in cell surface CCR7 and CD62L indicates that the majority of local CXCR5<sup>+</sup> T cells have recently entered the tonsillar tissue (see below).

Tonsillar CXCR5<sup>+</sup> T cells readily migrate in response to BCA-1, demonstrating that CXCR5 is functional. However, CXCR5 expression and responsiveness to BCA-1 are sensitive to T cell activation, as shown by polyclonal stimulation of tonsillar and circulating CXCR5<sup>+</sup> T cells. This contrasts strikingly with the migration responses to ELC and SDF-1, which are only observed late in CXCR5<sup>+</sup> T cell culture, well after enhancement of CCR7 and CXCR4 expression, illustrating that these two chemokine systems are not essential for the localization of CXCR5<sup>+</sup> T cells within tonsils. Failure in finding CXCR5<sup>-</sup> T cell lines and the inability to induce reexpression of this receptor in cell lines derived from sorted CXCR5<sup>+</sup> and CXCR5<sup>-</sup> T cells led us to propose that CXCR5 marks nonproliferating memory T cells. Consequently, CXCR5<sup>+</sup> T cells in peripheral blood may represent a subset of memory T cells that have left secondary lymphoid tissues before clonal expansion. The requirements for induction of CXCR5 expression in naive T cells are not defined. Preliminary data indicate that PHA or anti-CD3/CD28 stimulation are not sufficient, suggesting that CXCR5 expression in naive T cells requires contact with antigen-presenting cells. In sup-

port, CXCR5 expression during primary responses in mice was shown to depend on sequential signaling by CD28 and OX40 (40, 41).

How are circulating CXCR5<sup>+</sup> T cells recruited into tonsillar follicles? BCA-1 production is highly concentrated in the mantle zone, but is also found in the light zone of germinal centers, marking B cell areas with prominent T cell involvement. In agreement, follicular T cells colocalize with BCA-1-producing cells and express the same range of subset (CD4, CD45RO) and activation markers (CD69, HLA-DR, ICOS) that are present on CXCR5<sup>+</sup> T cells freshly isolated from tonsils. Evidently, CXCR5 expression characterizes the subset of tonsillar T cells that are attracted to areas with prominent BCA-1 production. Cellular sources of BCA-1 include reticular cells in the follicular mantle and follicular HEVs. Selective expression of BCA-1 in follicular as opposed to T zone HEVs may represent an important address code for direct recruitment of circulating CXCR5<sup>+</sup> T cells into B cell follicles. Similarly, SLC is selectively expressed on T zone (but not follicular) HEVs and is shown to direct the transmigration of CCR7-bearing T cells through induction of firm adhesion and subsequent diapedesis (30, 31, 38, 42). As such, BCA-1 and SLC are nonoverlapping in their sorting function and may therefore contribute to an efficient positioning of T cells with mutually exclusive properties within secondary lymphoid tissues. Peripheral blood B cells differ from the bulk of T cells in their uniform expression of both CXCR5 and CCR7. B cell recruitment into secondary lymphoid tissues of CXCR5<sup>-</sup> or CCR7-deficient mice is not impaired, suggesting that the absence of either chemokine receptor can be compensated by signaling through the other chemokine receptor (6, 13, 17, 30, 43). However, CXCR5 (but not CCR7) is critical for B cell follicle formation in the spleen and PPs (13, 17), which fits well with the follicular localization of CXCR5<sup>+</sup> T cells.

CXCR5<sup>+</sup> memory T cells are poor producers of cytokines. However, expression of ICOS strongly suggests that tonsillar CXCR5<sup>+</sup> T cells are engaged in B cell activation. This CD28-related costimulatory molecule is induced during T cells activation and is prominently expressed on T cells in the mantle and light zone of secondary follicles (44; and shown here). The B7-related ICOS ligand is expressed on B cells and macrophages and costimulates, together with TCR signaling, T cell proliferation (45–47). Hyperplasia in secondary lymphoid tissues, plasmacytosis, and elevated levels of blood IgG in transgenic mice that constitutively express soluble ICOS ligand point to a prominent role of this costimulatory system in proliferative and B helper responses of follicular T cells (46). A functional link between ICOS and CXCR5 expression is suggested here by the selective production of IgG and IgA during coculture of tonsillar CXCR5<sup>+</sup> T cells with B cells. Due to the preactivation status of tonsillar T cells, exogenous stimuli were not required for B cell stimulation, and lack of preferential IgM synthesis suggests that CXCR5<sup>+</sup> T cells are mainly active during secondary antibody responses. These effector functions together with the follicular localization demonstrate



that CXCR5<sup>+</sup> T cells play an important regulatory role in humoral immunity and led us to propose the term follicular B helper T cells (T<sub>FH</sub>) to designate this particular T cell subset.

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