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CXCR3 Is Induced Early on the Pathway of CD4\(^+\) T Cell Differentiation and Bridges Central and Peripheral Functions\(^1,2\)

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Chemokine receptors on T cells are frequently categorized as functioning either in immune system homeostasis within lymphoid organs, or in peripheral inflammation. CXCR3 is in the latter category and is reported to be expressed selectively on Th1 cells. We found that CXCR3 was expressed in vivo on newly activated tonsillar CD4\(^+\) T cells. Using CD4\(^+\) T cells from cord blood, we found that CXCR3 was induced by cellular activation in vitro independently of the cytokine milieu, although on resting cells, expression was maintained preferentially on those that had been activated in type 1 conditions. In inflamed tonsils, CXCR3*CD4\(^+\) T cells were localized around and within germinal centers. The inference that CXCR3 has a role in germinal center reactions was supported by the finding that the CXCR3 ligand CXC chemokine ligand 9 was expressed in a pattern demarcating a subset of germinal centers both in tonsil and in lymph nodes from an HIV-infected individual. We next investigated the role of CXCR3 on peripheral effector/memory CD4\(^+\) T cells by comparing its pattern of expression with that of CCR5, another Th1-cell associated chemokine receptor. Analysis of cells directly from peripheral blood and after activation in vitro suggested that CXCR3 expression preceded that of CCR5, supporting a model of sequential induction of chemokine receptors during CD4\(^+\) T cell differentiation. Taken together, our data show that CXCR3 can be expressed at all stages of CD4\(^+\) T cell activation and differentiation, bridging central function in lymphoid organs and effector function in peripheral tissues. *The Journal of Immunology, 2003, 171: 2812–2824.*

The chemokine system has been increasingly recognized as crucial to the organization of the immune system (1). An important component of this organization is the proper positioning and trafficking of lymphocytes. Changes in patterns of migration as lymphocytes mature and undergo activation and differentiation are essential if these cells are to contribute appropriately to primary and anamnestic immune responses. The complexity of the task confronting the adaptive immune system is reflected in the diversity of lymphocyte subsets, and this diversity includes multiple patterns of expression of a large number of chemokine receptors that regulate lymphocyte movement (2).

A basic division among mature lymphocytes is between the naive and memory/effector cells. The former are responsible for initial responses to novel challenges, and the latter mediate recall responses and effector functions. The differences in these cells’ histories is reflected in functional assays in vitro and in vivo as well as in patterns of expression of surface markers including adhesion molecules and chemokine receptors (3). These surface molecules mediate the profound differences in migration routes taken by naive and memory/effector cells. Although naive cells move among defined compartments within noninfamed lymphoid organs, memory/effector cells are equipped to infiltrate peripheral inflammatory sites (4, 5).

Chemokine receptors on lymphocytes have been divided between those mediating migration to lymphoid compartments, primarily for naive cells to maintain homeostasis in the absence of immune challenge, and those mediating migration of memory/effector cells to reactive sites (2). Chemokine receptors themselves have been used to define new T cell subsets (6, 7). In our previous work, we analyzed chemokine receptor expression and signaling on naive and memory human T cells both freshly isolated and after short-term activation in vitro. We found that one receptor, CXCR3, was unusual. Unlike other receptors expressed selectively on memory cells, such as CCR2, CCR3, and CCR5, CXCR3 could be induced to significant levels and was functional within 3 days after cross-linking the TCR of naive CD4\(^+\) T cells (8). CXCR3 is a receptor for CXC chemokine ligand 9 (CXCL9)\(^5\) (Mig), CXCL10 (IFN-γ-inducible protein 10), and CXCL11 (IFN-inducible T cell α chemotactrant), which are all inducible by IFN-γ (9, 10). CXCR3 has been reported by most (11, 12), but not all investigators (13) to be preferentially induced on Th1 as compared with Th2 cell lines derived in vitro, to be preferentially expressed on Th1 vs Th2 cells in peripheral blood (14, 15), and to be found on a high percentage of CD4\(^+\) T cells in type 1-dominated inflammatory processes (16). CXCR3 is also expressed on CD8\(^+\) T cells, on subsets of NK cells and peripheral blood B cells, and on a number of B cell malignancies (16–18). Data from both the analysis of

\(^5\) Abbreviations used in this paper: CXCL, CXC chemokine ligand; 7-AAD, 7-amino-acetoxy-methine-dye; CD40L, CD40 ligand; Tup, nonpolarized T; CCL, CC chemokine ligand.
human samples and mouse models of disease and organ transplantation have supported a role for CXCR3 and its ligands in type 1 inflammation in peripheral tissues (19–22).

Based on our previous work, we considered whether in humans CXCR3 might play a role not only on highly differentiated Th1 effector cells but also more generally on CD4+ T cells that had been recently activated. Our experiments described below support this hypothesis and widen our view of the roles of inflammatory chemokine receptors. Our data suggest that, with respect to the interrelated processes of CD4+ T cell differentiation, helper function, and sites of T cell localization, CXCR3 functions broadly—on cells that have been recently activated as well as those that are fully differentiated and on cells that participate in the germinal center reaction as well as those that infiltrate peripheral inflammatory sites. In addition, by comparing expression of CXCR3 and CCR5, we provide evidence that one organizing principle underlying expression of chemokine receptors is their sequential induction during the course of CD4+ T cell differentiation.

Materials and Methods

Reagents

All culture media, salt solutions, and FBS were obtained from Life Technologies (Gaithersburg, MD). The anti-CD3 mAb OKT3 was obtained from Ortho Biotech (Raritan, NJ), and the anti-CD28 mAb clone 9.3 was a gift of C. June (University of Pennsylvania, Philadelphia, PA). Anti-IL-4, anti-IL-12, and rIL-4 and rIL-12 were obtained from BD Pharmingen (San Diego, CA). rIL-2 was obtained from Hoffmann-La Roche (Nutley, NJ), and rIL-7 from PeproTech (Rocky Hill, NJ). Pyronin Y, 7-aminoactinomycin D (7-AAD), PMA, ionomycin, saponin, and phenylhydrazine were purchased from Sigma-Aldrich (St. Louis, MO). Monensin was purchased from Calbiochem (San Diego, CA).

Flow cytometry and immunofluorescence. mAbs against the following Ags were purchased from BD Pharmingen: CD4, CD11c, CD14, CD19, CD45RA, CD57, CCR5, CXC1, CXC2, CXC3, CXCR4, IFN-γ, IL-4, and Ki67. Abs against CCR1, CCR2, and CCR3 were purchased from R&D Systems (Minneapolis, MN). MφC63 mAb ascites (anti-CD19 for immunofluorescence) was a gift from T. Nutman (National Institute of Allergy and Infectious Diseases), and mAb 24-31 (anti-CD154) was a gift from R. Noelle (Dartmouth Medical School, Hanover, NH).

Cells and tissue

Human cord blood was collected from term placentas after delivery of healthy newborns at Shady Grove Adventist Hospital (Gaithersburg, MD). Buffy coats and elutriated lymphocytes from the Department of Transfusion Medicine, National Institutes of Health, were used for PBMC and subset isolation. Tonsils were obtained from either Children’s National Medical Center, National Naval Medical Center, and Shady Grove Adventist Hospital, as appropriate. Lymph nodes were obtained as part of a natural history study to evaluate tissue architecture and expression of HIV RNA and protein before and after antiretroviral therapy (23). All patients gave their informed consent to participate in the natural history study. The study protocol was reviewed and approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases.

For immunofluorescence, tonsils were embedded in OCT compound (Sakura Finetek, Torrence, CA) and snap frozen in a slurry of dry ice and isopentane. Sections were cut at a thickness of 10 μm and placed on gelatin-coated glass slides. For in situ hybridization, tonsils were cut into 2–4 mm slices and placed in 4% paraformaldehyde on an orbital rotator at room temperature for 72 h. The fragments were kept in 70% ethanol until they were embedded in paraffin and cut into 10-μm sections.

Mononuclear cells from cord blood, buffy coats, and tonsils were isolated by ficoll-Hypaque (Pharmacia, Piscataway, NJ) density centrifugation, and residual RBC were lysed with ACK lysis buffer (Biofluids, Rockville, MD). Tonsils were minced and passed through sieves before density centrifugation. Total tonsil leukocyte subsets were purified by digesting minced tonsils in RPMI 1640 with collagenase, hyaluronidase, and DNase (Sigma-Aldrich), and subjecting the resulting cell suspension to 50% Percoll (Pharmacia) density gradient centrifugation as previously described (24).

CD4+ T cells were isolated from cord blood with RosetteSep/human CD4+ T cell reagent (StemCell Technologies, Vancouver, BC, Canada). Tonsillar and peripheral blood CD4+ T cells were either isolated with RosetteSep reagent or by magnetic bead column selection (Miltenyi Biotec, Auburn, CA) according to the manufacturers’ protocols.

Culturing CD4+ T cells

For stimulating cord CD4+ T cells, on day 0, freshly isolated and purified CD4+ T cells were cultured at 1 × 106 cells/ml in 24-well plates in RPMI 1640/10% FBS with gentamicin (20 μg/ml; Life Technologies), and stimulated with plate-bound OKT3 (coating concentration, 10 μg/ml), soluble anti-CD28 (1 μg/ml), IL-2 (200 IU/ml), and either IL-4 (4 ng/ml) or IL-12 and anti-IL-4 (2 ng/ml and 0.4 μg/ml, respectively). On day 2, the medium was replaced with the medium replaced with fresh medium containing cytokines and mAbs. On day 3, the cells were harvested, washed, and resuspended at 1 × 106 cells/ml in fresh medium with IL-2, plus IL-4 or plus IL-12 and anti-IL-4. On days 3–6, the medium was changed as on day 2. On day 7, the cells were harvested again and resuspended in fresh medium with IL-2 and IL-7 (15 ng/ml). Thereafter, medium was changed every day but with one-half of the IL-2 of the previous day until days 16–19 when the cells were harvested, washed, and suspended in medium with IL-7 alone. Medium was then changed every day for 3–5 days at which time the cells were harvested and restimulated with plate-bound anti-CD3 and soluble anti-CD28, and the treatment protocol was repeated.

Cell staining, phalloidin assay, and flow cytometry

For phenotypic analysis of cell surface proteins, cells were stained with mAbs for 15 min on ice (staining for CCR5 was done at room temperature) in 1% FBS with Ca2+ and Mg2+, washed three times, and fixed in 0.8% parafomaldehyde. For cytokine analysis, cells were stimulated for 4–6 h with PMA (20 ng/ml) and ionomycin (1 μM) in the presence of monensin (2 μM). Cells were then fixed in 4% paraformaldehyde, washed, and stained as previously described (25). For cell cycle analysis (data not shown), mAb-stained cells were suspended in citrate/phosphate buffer containing BSA (0.5%), saponin (0.004%), and EDTA (5 mM), and incubated with 7-AAD (20 mM) at room temperature for 30 min, followed by pyronin Y (5 μM) on ice for 10 min (26).

To detect rapid actin polymerization, tonsillar mononuclear cells were stained for phenotypic markers at room temperature, washed, and stained at 37°C for 10 min at 1.25 × 106 cells/ml in HBSS/1% FBS with Ca2+ and Mg2+. Chemokines were added at a final concentration of 1 μg/ml, and the cells were vortexed. At various time points, a solution of FITC-phalloidin and unconjugated phalloidin (0.8 U/ml and 5 μg/ml, respectively; Molecular Probes, Eugene, OR), saponin (0.1%), and 23% paraformaldehyde in PBS was diluted 1/5 into the sample of chemokine-stimulated cells. The fixed and permeabilized cells were then incubated on ice for 10 min, washed two times in PBS with 1% BSA and 0.1% saponin, and resuspended in PBS.

Ab staining, phalloidin, and cell cycle data were collected on a FACS-Calibur (BD Biosciences, San Jose, CA). For the time course analyses, 10,000 events were collected. Cell cycle and phalloidin staining were quantified manually with MACS (Miltenyi Biotec, Auburn, CA) and a flow cytometry software program. Tonsillar mononuclear cells, 100,000–300,000 events were collected. Cell sorting was done on a FACSVantage flow cytometer (BD Biosciences). Flow cytometry data files were analyzed using FlowJo software (Tree Star, San Carlos, CA) for the Apple (Cupertino, CA) Macintosh.

Chemotaxis

Tonsillar mononuclear cells were suspended in RPMI 1640, 0.5% BSA, 10 mM HEPES at a concentration of 1 × 106 cells/ml, of which 100 μl was placed in the insert of a Transwell chamber (pore size, 5 μm; Costar, Corning, NY). The same medium containing chemokine (600 ng/ml) was placed in the lower well, and the cells were incubated at 37°C in 5% CO2. After 3 h, the cells in the lower chamber were harvested, counted with a hemocytometer, stained for CD19, CD4, CD45RA, and CD45RO, and analyzed by flow cytometry. Chemotaxis experiments with cord blood CD4+ T cells were performed identical except that the starting concentration was 5 × 105 cells/ml; the incubation time was 2 h, and the cells were stained for CD4, CD45RA, CD45RO, and CXCR3.

Immunofluorescent staining of tonsils

Frozen sections were fixed in acetone at −20°C for 20 min and allowed to dry. Endogenous peroxidase was inactivated with the irreversible inhibitor phenylhydrazine (1 mg/ml) for 5 min (27). The tyramide signal amplification system (TSA-Direct kit; NEN Life Science Products, Boston, MA) was used for staining with FITC, Cy3, and Cy5. Primary Ab staining was

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for 1 h at room temperature, followed by three washes in PBS, followed by
the secondary HRP-conjugated reagents: anti-mouse IgG1, anti-mouse
IgG2a, anti-mouse IgG2b (all from Southern Biotechnology, Birmingham,
AL), anti-FITC (DAKO, Carpinteria, CA), and avidin (BD PharMingen).
Between each stain, HRP was inactivated with phenylhydrazine. When
costaining for CXCR3 and CD154 (both unconjugated mouse IgG1 mAbs),
the section was acid washed between the two stains (PBS; pH 3) for 3 min
to dissociate Ag-Ab complexes. Some sections were then treated with an
IgG1 isotype control in place of the second primary Ab, followed by the
anti-mouse IgG1 secondary Ab as a negative control in part to insure that
the acid wash completely removed Ab from the first stain. After staining
for surface Ags, sections were stained with Hoechst 33482 (Molecular
Probes) for morphologic de
finition, and the slides were mounted with
Vectashield (Vector Laboratories, Burlingame, CA).

Slides were analyzed on a Leica (Deerfield, IL) TCS-NT/SP confocal
microscope using a ×20 objective. Serial 1-μm-thick slices were analyzed
throughout the depth of the tissue section, and from the stack of image
slides, three contiguous images were combined into one using the Imaris
graphics program (Bitplane, Zurich, Switzerland). The resulting image was
edited with Photoshop 5.5 (Adobe Systems, San Jose, CA).

In situ hybridization

Hybridizations were done on sections from paraffin-embedded tissue using
35S-labeled antisense and sense riboprobes. The probes for CXCL9 were
made from a fragment containing bp 1217–1810 of GenBank sequence
with accession no. X72755, for CXCL10 from the fragment containing bp
22–720 of GenBank sequence with accession no. X02530, and for

FIGURE 1. CXCR3 is induced on recently activated CD4+ T cells in vivo. Abbreviations of fluorophores for all figures are as follows: APC, allophycocyanin;
FITC; PE; PECy5, PE-Cy5 tandem conjugate. A, Isoforms of CD45 define naïve (CD45RA+CD45RO−), transitional (recently activated, CD45RA+CD45RO−),
and memory (CD45RA−CD45RO+) subsets on tonsillar CD4+ T cells. Only CD4+ T cells are shown. B, CXCR3 and CCR5 on subsets of tonsillar CD4+ T cells.
Histograms show staining of naïve, transitional, and memory CD4+ T cells with Abs against the chemokine receptors as noted (bold lines) as compared with
isotype-matched control Abs (dashed lines). The data in A and B are representative of 15 experiments. C, CXCR3 is functional on tonsillar CD4+ T cells. Tonsillar
mononuclear cells were used for a Transwell chemotaxis assay. The results were obtained by counting total cells and by analysis by flow cytometry after staining
cells for CD4. The concentration of chemokines in the lower chamber was 1 μg/ml. These data are representative of two experiments. D, CXCR3 is functional
on transitional cells produced in a type 1 environment in vitro. Cord CD4+ T cells were stimulated for 3 days with anti-CD3, anti-CD28, and IL-12 as in Fig. 2A.
Chemotaxis assays were done as above. These data are representative of three experiments. E, CXCR3 is functional on tonsillar transitional cells. Tonsillar
CD4+ T cells were stained with fluorescent conjugated Abs against CD4, CD45RA, and CD45RO, and treated with 1 μg/ml chemokine, after which they were fixed
and stained with FITC-phalloidin for polymerized (F-)actin. Phalloidin staining after 5 s of exposure to chemokine (solid line) vs untreated cells (dashed line) is shown.
Cells within the transitional subset responded to CXCL9. Responses to CXCL12 and CXCL8 (higher than for CXCR3 and none, respectively) are shown for
comparison. These data are representative of three experiments.
CXCL11 from the fragment containing bp 25–1391 of GenBank sequence with accession no. AF002985. A CXCL9 fragment was produced from a cDNA template (28) by PCR and ligated to T7 promoter sequences with reagents and protocols from the Lig’n’scribe kit (Ambion, Austin, TX). Fragments for making antisense and sense riboprobes were made by PCR using CXCL9-specific primers and Lig’n’scribe PCR Adapter Primer 2 as supplied. For CXCL10, the fragment was isolated from a cDNA clone (28) and inserted into the EcoRI site of pBluescript SKII (Stratagene, La Jolla, CA). Riboprobes were made using T3 RNA polymerase from the CXCL10 sequences in both orientations in plasmids cut with BamHI. For CXCL11, the fragment was in IMAGE clone no. 1058310, in vector pITT3D-PucI, and was kindly supplied by J. Smith (University of California, Los Angeles, School of Medicine, Los Angeles, CA). The antisense probe was made using T3 RNA polymerase from the plasmid cut with XhoI, and the sense probe was made using T7 RNA polymerase from the plasmid cut with NotI. In situ hybridizations were performed as previously described by Molecular Histology (Gaithersburg, MD) according to their published protocols (29), and sections were stained with H&E.

Quantitative RT-PCR
RNA from sorted cells was isolated using ion exchange columns (RNaseasy; Qiagen, Valencia, CA). For isolation of RNA from tonsils, tissue was placed in TRIzol reagent (Life Technologies) and homogenized with a Polytron (Kinematica, Lucerne, Switzerland), and RNA was isolated according to the manufacturer’s protocol. First-strand cDNA synthesis was performed on ≤5 μg of RNA per reaction using SuperscriptII (Life Technologies) with oligo(dT) primers following the manufacturer’s instructions.

Real-time quantitative RT-PCR was performed using the TaqMan assay and PCR amplifications were performed in an Applied Biosystems (Foster City, CA) Prism 7700 sequence detection system. CXCL9 primers and probes were gifts from G. Schwartz (National Cancer Institute, Bethesda, MD) (30), and the probe/primer set for GAPDH was purchased from Applied Biosystems. Other primer sequences were as follows: CXCL10, 5′-CGATTCTGTGTTGCTGCTATT3′ and 5′-CAGGATATTTTCTCAAGGTT3′; and CXCL11, 5′-CTCTGCTGTGATATTGTGGC3′ and 5′-CAGGCCATTATCAAGAGCA3′. Other probe sequences were as follows: CXCL10, 5′-TTTCTGACCTTAAAGGCTCATC3′; and CXCL11, 5′-CAGTTGTCAAGGCTTCTC3′. The CXCL9 probe was labeled at the 5′ end with FAM (excitation/emission λmax = 494/518 nm) and the CXCL10 and CXCL11 probes were labeled with the reporter dye VIC (excitation/emission λmax = 538/552 nm), and the GAPDH probe was labeled with JOE (excitation/emission λmax = 560/582 nm). Primers and probes for CXCL10 and CXCL11 were designed using Primer Express software (version 1.0; Applied Biosystems).

For each primer/probe set, cDNA from IFN-γ-stimulated monocyte-derived macrophages or THP-1 cells were serially diluted 1/10 and amplified in duplicate for standard curves. Linear-regression R² values for each curve between 17 and 32 cycles were from 0.965 to 0.995 (not shown). Relative quantification of chemokine mRNAs in leukocyte subsets was done using Sequence Detector software (version 1.7; Applied Biosystems), and normalized to expression of the housekeeping gene GAPDH.

Results
Expression of CXCR3 on CD4+ T cells activated in vivo
Because our earlier data demonstrated induction of CXCR3 on naive CD4+ T cells after activation in vitro (8), we investigated the expression of CXCR3 on CD4+ T cells that had been recently activated in vivo. Although such cells are rare in peripheral blood, higher numbers are found in human tonsils. To identify the naive tonsillar CD4+ T cells that had been recently activated, we analyzed the expression of CD45 isoforms as shown in Fig. 1A. Although naive and memory cells are single positive for CD45RA and CD45RO, respectively, the recently activated transitional cells express both CD45RA and CD45RO (31). Fig. 1B shows that significant subsets of CXCR3+ cells were found in both the transitional and memory CD4+ T cell populations. For comparison, we also analyzed the expression on tonsillar T cells of a number of chemokine receptors, including CCR5, which we found on a small percentage of transitional cells. Not shown are data on CCR1 and CCR2, which we did not detect on significant numbers of tonsillar

![Graph](http://www.jimmunol.org/)

**FIGURE 2.** CXCR3 expression is activation associated on cord blood CD4+ T cells stimulated and polarized in vitro. A, CXCR3 is induced during activation on both IL-4-treated and IL-12- plus anti-IL-4-treated cells, but induction is more uniform and persists at rest on cells treated with IL-12. The experimental design is depicted graphically below the histograms. Cells were treated with two cycles of plate-bound anti-CD3 (10 μg/ml), soluble anti-CD28 (1 μg/ml), IL-2 (200 IU/ml), IL-4 (4 ng/ml), or IL-12 plus anti-IL-4 (2 ng/ml and 0.4 μg/ml, respectively), and IL-7 (15 ng/ml) during the times indicated by the open bars. The IL-2 dose was reduced as indicated by the tapered bar. The exact number of days of the last phase of each cycle (IL-7 alone) was at least 3 days, but varied slightly among experiments. The histograms show staining with anti-CXCR3 (bold) or isotype control (dashed) of fetal T cells activated in vivo.

B, CXCR3 is expressed on cells continuously activated in the presence of IL-4. Cord blood CD4+ T cells were maintained in IL-4 and IL-2 rather than weaning IL-2 in the presence of IL-7 (compare with days 19 and 44 in A). These data are from the same experiment as Fig. 2A and are representative of two experiments.
CD4⁺ T cells, and CCR3, which, as for CCR5, we found on a small subset of transitional cells. Thus, CXCR3 was expressed on transitional CD4⁺ T cells at a frequency higher than was seen for a panel of other T cell chemokine receptors associated with peripheral inflammation.

To verify that CXCR3 on tonsillar T cells was functional, we performed chemotaxis assays. As shown in Fig. 1C, tonsillar CD4⁺ T cells responded to CXCL9, although in lower numbers than to CXCL12, which is not surprising given the large percentage of T cells that express the CXCL12 receptor, CXCR4. As anticipated from the pattern of CXCR3 expression and the composition of the starting population, almost all of the responding CD4⁺ T cells were CD45RO⁺CD45RA⁻ (data not shown). Because the number of CD45RO⁺CD45RA⁻CD4⁺ T cells in tonsil was too low for them to be analyzed for chemotaxis, we tested CD45RO⁺CD45RA⁺CD4⁺ T cells (flow cytometry data not shown) that we derived from human cord blood after 3 days of activation in vitro under type 1 polarizing conditions (see below). As shown in Fig. 1D, these cells migrated well to CXCL9, in numbers comparable to those for CXCL12. Finally, we tested tonsillar CD45RO⁺CD45RA⁺CD4⁺ T cells directly ex vivo for responses to CXCL9 using FITC-phalloidin to detect polymerized

FIGURE 3. Location of CXCR3⁺CD4⁺ T cells in human tonsil. Acetone-fixed frozen sections (10 μm thick) were stained and analyzed by confocal fluorescent microscopy. Images from three consecutive layers (1 μm each) were combined to produce the micrographs shown. All images were acquired at ×200. Stains using isotype control Abs were black (not shown). A, CD4⁺CXCR3⁺ cells are found adjacent to B cell follicles and in germinal-center light zones (arrows). The section was stained for Ki67 (FITC; shown as green), CXCR3 (Cy3; shown as red), and CD4 (Cy5; shown as blue). Ki67⁺ cells identify the germinal-center dark zones. CD4⁺CXCR3⁺ cells are violet. For comparison, six color controls of an area at the edge of a germinal center and identified by the arrowhead are shown: three single-color images on the left and three two-color images on the right subtracting blue, red, and green (top to bottom, respectively). This section is representative of those from four donors. B, Coexpression of CXCR3 and CD154 (CD40L). The section was stained for CD19 (FITC; shown as green), CXCR3 (Cy3; shown as red), and CD154 (Cy5; shown as blue). The CD19⁺ cells identify a germinal center. CXCR3⁺CD154⁺ cells are violet. Color controls are as in A and correspond to the area in the white box. The blue (CD154) and red (CXCR3) are not perfectly coincident, because CD154 is intracellular (34) and CXCR3 is on the cell surface. This section is representative of those from five donors. C, CXCR3⁺ memory CD4⁺ T cells coexpress CD57. Tonsillar mononuclear cells were stained for CD57, CD4, CD45RO, and CXCR3. Staining for CXCR3 and CD57 is shown for the CD45RO⁻ (memory) CD4⁺ T cells. The same pattern was found with cells from five donors. D and E, CD4⁺CD45RA⁺CXCR3⁺ T cells are in the T cell zone. The section was stained for CD45RA (FITC; shown as green), CXCR3 (Cy3; shown as red), and CD4⁺ (Cy5; shown as blue). The triple-positive cells are white. Color controls as in A correspond to area below the arrowhead. These sections are representative of those from three donors. D, Two germinal centers (GC) and the T cell zone (T) between them are shown. E, The T cell zone, including CD4⁺CD45RA⁺CXCR3⁺ cells is magnified for better visualization.
FIGURE 4. (Figure legend continues)
(F-actin by flow cytometry (32). As shown in Fig. 1E, a subset of transitional cells responded to stimulation with 1 μg/ml the CXCR3 ligand CXCL9. CXCL12 and CXCL8 served as positive and negative controls, respectively. Taken together, these data demonstrate that CXCR3 expressed on memory and on recently activated CD4+ tonsillar T cells is functional.

Expression of CXCR3 on naive CD4+ T cells activated in vitro

The data from the tonsillar cells supported an association between recent activation of CD4+ T cells in vivo and induction of CXCR3. We sought to investigate this relationship more extensively under controlled conditions in vitro, starting with CD4+ T cells from human cord blood, which are uniformly naive. We developed a protocol as shown in Fig. 2A, in which cells could be put through cycles of activation and rest (or activated with IL-2 continuously). Because of the reported preferential expression of CXCR3 on Th1 cells (11, 12), we activated cells in defined type 1 or type 2 environments. We stimulated the cord blood CD4+ T cells (≥98% pure, not shown) with plate-bound anti-CD3 and soluble anti-CD28 for 3 days in the presence of IL-2 and polarizing cytokines (IL-4 vs IL-12), and continued the polarizing cytokines until day 7, a time point chosen because the cells had passed peak proliferation (not shown), and they had completed moving from naive (CD45RA+/CD45RO−) to transitional (CD45RA+/CD45RO−) to memory (CD45RA−CD45RO+) phenotype (not shown). The polarizing cytokines were then stopped, and the IL-2 was weaned in the presence of IL-7 over 7–10 days. We used IL-7 because it is a homeostatic cytokine that protects T cells from programmed cell death (33). Cell cycle analysis with 7-AAD and pyronin Y demonstrated activation-induced cycling in >85% of the cells and a return to G0 for ~80 and 50% of the cells after the first and second rounds of stimulation, respectively (not shown). The lymphocyte morphologies were consistent with their staining, with aggregated and irregularly shaped cells associated with active cycling, and noncycling cells appearing round and not clumped (not shown). Cells stimulated in the type 1 or type 2 environment developed the expected cytokine profiles as shown by intracellular staining (see Fig. 7A).

Fig. 2A shows that, within 1 day, CXCR3 was expressed on a small fraction of cells in the type 2 (IL-4) and the type 1 (IL-12) environments. At 3 days, when the CD4+ T cells consistently expressed both CD45RA and CD45RO (transitional cell phenotype, not shown), the percentage of CXCR3+ cells peaked in both the type 1 and 2 environments. Although the cells stimulated in the presence of IL-12 retained high expression of CXCR3 (Fig. 2A, day 7), expression was lost on those cells stimulated in the type 2 condition. At rest (day 19), a small subset of the cells stimulated in the presence of IL-4 expressed CXCR3, compared with expression on all IL-12-stimulated cells. Similar results were obtained during the second round of stimulation, although the fraction of cells in the type 2 environment expressing CXCR3 at rest (day 44) was larger than after the first round.

Because expression of CXCR3 in the type 2 cells decreased after withdrawal of IL-4 and weaning of IL-2, we asked whether cells maintained in type 2-activating conditions might be driven to high expression of CXCR3. Cells kept in IL-2 and IL-4 over the course of either a primary (Fig. 2B, left) or secondary (right) stimulation expressed high levels of CXCR3, comparable with those cells stimulated in the presence of IL-12 seen in A. The cells maintained in IL-4 and IL-2 and those cultured with IL-4 and then brought to rest with IL-7 had similar patterns of intracellular cytokine staining: >25% of the cells were IL-4+ IFN-γ (not shown). Thus, the increases in CXCR3 expression on cells kept in activating vs resting conditions occurred without change in cytokine profiles. However, even after prolonged stimulation in type 2 conditions, when the IL-4 was withdrawn and IL-2 was weaned, CXCR3 expression was significantly reduced (not shown). Taken together, Figs. 1 and 2 show that CXCR3 is expressed as follows: 1) during primary activation when cells are undergoing naive to memory transition both in vivo and in vitro, 2) on recently activated cells regardless of whether the activation occurs in a type 1 or type 2 environment, 3) on chronically stimulated cells regardless of their cytokine phenotype, and 4) on cells that were activated in type 1 conditions regardless of their state of activation, in contrast to the resting CD4+ T cells that had been activated in a type 2 environment, upon which CXCR3 expression is not maintained.

Localization of CXCR3+ CD4+ T cells in tonsil

Expression of CXCR3 on recently activated as well as memory CD4+ tonsillar T cells raised the possibility that CXCR3+ cells might be playing a role in Th function at this site. We addressed this question by localizing CXCR3+CXCR3+ T cells within the tonsil, by analyzing the coexpression of CXCR3 and other markers, including those associated with follicular helper cells, and by identifying sites and cells expressing CXCR3 ligands.

In the confocal images in Fig. 3A, the centroblasts in the germinal-center dark zones are identified by their expression of the proliferation-associated Ag Ki67. CXCR3 staining is seen on CD4+ cells at the edges of the germinal centers and within the germinal-center light zones. CD4+ T cells surrounding and within germinal centers have been shown to have a memory (CD45RO+) phenotype (Ref. 34 and data not shown). The percentage of germinal centers (Ki67+ follicles) with a pattern of CXCR3 staining similar to that shown in Fig. 3A varied among the tissues analyzed from four donors (range, 23–62%; mean ± ΔD, 41.6 ± 16). Studies of human tonsils have shown that T cells at the edge of the T cell zone and adjacent to germinal centers also express preformed CD40 ligand (CD40L) (CD154), supporting a role for these cells in providing help to B cells (34). Staining sections of tonsils for CXCR3 and CD154 revealed that a subset of CD154+ cells at the margins of the T cell zones coexpressed CXCR3 (Fig. 3B). To buttress our finding of CXCR3+ cells within germinal centers, we
analyzed CD4<sup>+</sup>CD45RO<sup>+</sup> cells by flow cytometry for coexpression of CXCR3 and CD57, an Ag that in tonsils is expressed specifically on germinal-center T cells (6, 35). As seen in Fig. 3C, 15–20% of the CXCR3<sup>+</sup>CD4<sup>+</sup> memory T cells in the tonsil also expressed CD57.

Finally, we sought to identify the small number of tonsillar transitional, CXCR3<sup>+</sup> cells in tissue sections by finding CXCR3<sup>+</sup>CD4<sup>+</sup> cells that had not yet lost expression of CD45RA. These cells could be identified occasionally in clusters within T cell zones (Fig. 3, D and E). Together, these data establish that CXCR3 is expressed on subsets of tonsillar CD4<sup>+</sup> T cells that have recently been activated and on some CD4<sup>+</sup> T cells that by location and expression of relevant markers are associated with providing B cell help.

**Expression of CXCR3 ligands in tonsil and lymph node**

Although the above data are compatible with a functional role for CXCR3 on CD4<sup>+</sup> T cells within tonsils, such a role would require expression of one or more CXCR3 ligands in situ. Fig. 4 shows bright (A)- and dark (B-D)-field views of a tonsil section probed for mRNA for the CXCR3 ligand CXCL9. The dark-field images show intense foci of expression in the interfollicular (T cell) zones and, of particular interest, at the borders of some germinal centers (Fig. 4, B and D). Hybridization for CXCL10 showed foci of signal in interfollicular regions, but the extent and intensity of the signal was less than for CXCL9, and no hybridization was seen at the germinal center borders (not shown). No signal was seen after hybridizing with a probe for the third CXCR3 ligand, CXCL11 (not shown).

To determine whether the pattern of CXCL9 expression in the tonsil was generally true for reactive lymphoid tissues, we performed hybridizations on sections of lymph nodes removed from an HIV-1-infected individual before and after antiretroviral therapy (Ref. 23 and see Materials and Methods). Hybridizations for HIV-1 RNA showed extensive signal in the pretreatment specimen that had all but disappeared in the lymph node taken after therapy (not shown), although the latter specimen remained hyperplastic with persistent HIV-1 Ag (not shown) and multiple germinal centers. As shown in Fig. 4, E, F, and H, there was dramatic expression of CXCL9 mRNA in the interfollicular/paracortical regions of the lymph node removed before therapy, and here again, as in the tonsil, expression was seen both in T cell zones and ringing germinal centers. In this lymph node more than in tonsil, CXCL9 expression was also found in foci within germinal centers (Fig. 4H). Following antiretroviral therapy, the pattern of CXCL9 expression was similar, although the intensity of the CXCL9 signal was diminished. In this latter specimen, signal was found both in the interfollicular regions and concentrated at the borders as well as within what were often bizarrely shaped germinal centers (Fig. 4G).

To characterize the cell types expressing CXCL9 and CXCL10 in the tonsil, we fractionated tonsil leukocytes and analyzed expression of CXCL9 and CXCL10 mRNA by real-time PCR (TaqMan). Fig. 5 shows that, among cells isolated by Percoll density centrifugation, dendritic cells (CD11c<sup>+</sup>CD14<sup>+</sup>) and, to a greater extent, macrophages (CD11c<sup>+</sup>CD14<sup>+</sup>) were the primary sources of CXCL9 and CXCL10 mRNA. By RT-PCR, levels of expression of CXCL9 relative to GAPDH were ~10-fold that of CXCL10, which in turn was ~10-fold that of CXCL11 (not shown). Although one cannot extrapolate precise ratios of expression of the three different ligand genes from these results, they are consistent with the relative levels of signal for these mRNAs seen in the tonsil by in situ hybridization. The prominent expression of CXCL9 by tonsillar APC and at sites where CXCR3<sup>+</sup>CD4<sup>+</sup> T cells are found—in T cell zones where T cells first become activated and at edges of germinal centers where helper memory cells aggregate—is strong presumptive evidence for a functional role for CXCR3 at these sites.

**Comparison between CXCR3 and CCR5**

Published data demonstrate the preferential expression of CXCR3 on Th1 cells in peripheral blood and a role for CXCR3 and its ligands in peripheral type 1 inflammation. Our in vitro results (Fig. 2) are consistent with these findings, because among our resting memory cells, only those activated in the type 1 environment continued to express CXCR3. Having established that CXCR3 is induced early after activation on naive CD4<sup>+</sup> T cells—and concluded that the receptor might be playing a role on these cells within lymphoid organs—we next sought to define more precisely the role of CXCR3 on the downstream memory/effecter CD4<sup>+</sup> T cells. The early induction of CXCR3 suggested that this and other inflammatory chemokine receptors might be induced sequentially.
during CD4⁺ T cell differentiation. We investigated this question further by comparing patterns of expression for CXCR3 with those for CCR5, which like CXCR3 is expressed on memory CD4⁺ T cells and has been reported to be expressed preferentially on Th1 cells (36). Previous data from our and other laboratories have shown a bias for CCR5 expression on the CD62L⁻ and CD62L⁺ subsets (7, 8, 37) memory CD4⁺ T cell subsets. By contrast, we have reported more balanced expression of CXCR3 on the CD62L⁻ and CD62L⁺ subsets (8), and analogous findings have been reported for CXCR3 on the subsets defined by CCR7 (7). Based on the distinctions and precursor-product relationship between the central (CD62L⁻CCR7⁺) and effector (CD62L⁻CCR7⁻) memory cells as postulated by Sallusto et al. (7), these data would suggest that CCR5 is generally expressed on cells that are more highly differentiated as compared with cells expressing CXCR3 alone.

We thus determined patterns of coexpression of CXCR3 and CCR5 on CD4⁺ T cells from peripheral blood and tonsils and investigated the functions of the appropriate subsets by analyzing their cytokine profiles. Fig. 6A shows that CCR5⁺CD4⁺ T cells are found predominantly within the CXCR3⁺ subset, consistent with the induction of CCR5 during additional differentiation of CXCR3⁺ cells. The CD4⁺ T cell subsets defined by CXCR3 and CCR5 were sorted and stained for intracellular cytokines after short-term activation with PMA and ionomycin. Fig. 6A shows restriction of IFN-γ⁺IL-4⁻ and IFN-γ⁺IL-4⁻ cells to CXCR3⁻ and CXCR3⁺ subsets, respectively, on the cells from peripheral blood, consistent with published data (13, 14), and shows a similar result for CD4⁺ T cells from tonsil. In comparing the CXCR3⁺CCR5⁻ and the CXCR3⁺CCR5⁺ subsets, three important differences are shown. First, the IFN-γ⁺IL-4⁻ cells in the CXCR3⁺CCR5⁻ subset expressed higher levels of IFN-γ than those in the CXCR3⁺CCR5⁺ subset. Second, the CXCR3⁺CCR5⁻ subset included more IFN-γ⁺IL-4⁻ (Th0) cells than its CCR5⁻ counterpart, and third, the CXCR3⁺CCR5⁻ subset included more cytokine-negative (nonpolarized T (Tnp)) cells than in the CXCR3⁺CCR5⁺ subset. Fig. 6B shows data quantified from seven experiments demonstrating that these results were consistent and statistically significant.

Published data (38, 39) suggest that the cytokine profile that we found for the CXCR3⁺CCR5⁺ subset is an indication that these cells had undergone additional activation/differentiation events as compared with the cells that were CXCR3⁺CCR5⁻. To validate, using our system, the changes in cytokine patterns reported with CD4⁺ T cell differentiation, we followed cytokine profiles during two rounds of activation by the protocol diagrammed in Fig. 2A. Fig. 7A shows a higher percentage of IFN-γ⁺ cells, a lower percentage of Tnp cells, and in the IL-12 stimulated cells, a significant Th0 subset after the second (day 38) as compared with the first (day 23) stimulation. Together, these data are consistent with CCR5 marking cells further down the pathway of Th1 cell proliferation/differentiation as compared with CXCR3. We demonstrated sequential induction of these receptors directly by following the expression of both CXCR3 and CCR5 during a single round of T cell activation and rest in vitro as shown in Fig. 7B. CCR5 was induced later than CXCR3, and like CXCR3, CCR5 was induced preferentially on cells activated in the type 1 environment.

Discussion

Our initial observations on the early expression of CXCR3 after activation of naive CD4⁺ T cells (8) led us to investigate in more detail the relationship between CXCR3 and CD4⁺ T cell activation. In so doing, we sought to characterize unique aspects of CXCR3 among inflammatory chemokine receptors expressed on lymphocytes and use CXCR3 to reveal general patterns of chemokine receptor expression during CD4⁺ T cell differentiation. Our major findings are the following: 1) CXCR3 is induced during primary activation when cells are undergoing naive-to-memory transition both in vivo and in vitro, 2) although induction occurs with activation independently of the cytokine milieu, CXCR3 expression is stable only on cells that have been activated in a type 1 environment, 3) CXCR3 and its ligands are expressed by cells and at sites within the tonsil that suggest a role for this receptor/ligand group within lymphoid organs, perhaps in providing T cell help to B cells, and 4) CCR5 is expressed on a subset of CXCR3⁺CD4⁺ T cells that have in general, as compared with...
CXCR3+CCR5+ cells, undergo additional activation/proliferation in vivo, which suggests sequential induction of CXCR3 and CCR5 during Th1 cell differentiation. From these data, we propose that CXCR3 is an inflammatory receptor that functions on memory CD4+ T cells throughout the pathway of CD4+ T cell activation and differentiation and that acts not only in the recruitment of effector cells to peripheral sites but also within lymphoid tissue.

We found that CXCR3 was expressed on recently activated tonsillar CD4+ T cells and was beginning to appear on some cells as early as 24 h after primary activation in vitro in both type 1 (IL-12 and anti-IL-4) and type 2 (IL-4) environments. It is of note that, although CXCR3 could be induced on all CD4+ T cells under some activation protocols in vitro (Fig. 2), only a subset of recently activated tonsillar CD4+ T cells were CXCR3+. Failure to detect CXCR3 on all the tonsillar cells is not surprising given the heterogeneity in activating conditions in vivo. For example, because activation in the tonsil was not synchronous, some CD45RO+CD45RA+ cells may not have up-regulated CXCR3 yet, and some exposed to activating factors for a limited time in a type 2 environment may only weakly or transiently express CXCR3 (see Fig. 2A, first-round stimulation). Although in our in vitro experiments expression of CXCR3 was stable after withdrawal of IL-2 only on cells that had been activated in type 1 conditions, high levels of CXCR3 could be induced on Th2 cells by continued culturing in IL-4 and IL-2.

The expression of CXCR3 on activated/transitional (and memory) CD4+ T cells within tonsils suggested that the receptor might have a role on these cells within lymphoid tissues. Expression of the CXCR3 ligand CXCL9 in the tonsil of a child and in lymph nodes taken from an HIV-1-infected adult provides strong presumptive evidence to support this hypothesis. In situ hybridizations revealed expression not only in foci within T cell regions, but also along the edges of, and occasionally within germinal centers (Fig. 4). To our knowledge, a circumferential pattern of chemokine expression at germinal-center margins has not been previously described. By real-time RT-PCR, expression of CXCL9, and at a lower level CXCL10, was detected in tonsillar macrophages and dendritic cells (Fig. 5). Tonsil and lymph node sections stained for macrophage markers (Ref. 40 and our unpublished observations) show these resident cells to be widely distributed throughout lymphoid tissue including T cell zones and germinal centers, i.e., regions where CXCL9 expression was detected by in situ hybridization. Our findings in tonsil are not surprising, given the previous demonstrations of CXCL9 expression in macrophages and/or dendritic cells (19, 28, 41). Taken together, these data demonstrate that CXCR3 ligands are expressed by small numbers of macrophages and dendritic cells at scattered sites, presumably associated with foci of activated T and in some cases B cells.

Consistent with a functional role for its ligand(s), CXCR3 was found on CD4+T cells both within the T cell zones and aggregated around and sometimes within germinal centers (Fig. 3). In addition, CXCR3+ cells were found to coexpress CD57, a marker specific for germinal-center T cells (6, 35). Memory CD4+ T cells at the edge of the T cell zone adjacent to germinal centers have been shown to express CD40L (CD154) (34), which is critical for mediating T cell help for activated B cells, and we also showed by immunofluorescence that some of the CXCR3+CD4+ T cells at these sites contained CD154. Together, these data suggest that CXCR3 and its ligands may have roles in supporting T cell activation within the T cell zones and in the function of activated/memory Th cells that surround germinal centers. CXCR3 ligands could function within microcompartments (in concert with constitutively expressed chemokines such as CC chemokine ligand 19 (CCL19), CCL21, and CXCL13 (42–46)) to alter the type of response, for example through the preferential recruitment or retention of Th1 cells. CXCR3 may be important in optimizing T cell-APC interactions at these sites. It has recently been reported, for example, that CXCL10 is essential for maintaining dendritic cell-Th1 cell clusters within reactive lymph nodes in mice (47). And if CD4+ T cells require sequential and/or repeated encounters with Ag-bearing APC for continued and effective activation (48), CXCR3 and its ligands may play a role in this process. Direct effects of CXCR3 on T cell activation are also possible, as suggested by the report of decreased proliferation of T cells from CXCR3-deficient mice in an MLR (20).

The association of CXCR3 and its ligands with germinal-center reactions suggests a role in optimizing Ab responses. In support of this possibility, Ab responses to a bacterial pathogen are diminished in CXCL9-deficient mice (49), and reduced Ab responses to

FIGURE 7. CXCR3 and CCR5 are expressed sequentially during CD4+ T cell activation/differentiation. A, The cytokine profiles of cord blood CD4+ T cells change after a second round of activation and rest. Cord CD4+ T cells were subjected to two rounds of stimulation as in Fig. 2A. The Th0 (IFN-γ+IL-4−) subset is larger when the cells were stimulated in the type 1 (bottom) vs type 2 (top) environments, and after the second (right) vs the first (left) round of stimulation. Anti-IL-4 was conjugated to PE or to Cy5PE, the latter of which gave a higher staining background.

These data are representative of two experiments. B, CCR5 is expressed after CXCR3 in primary activation of cord blood CD4+ T cells. Cord blood CD4+ T cells were stimulated and brought to rest as in Fig. 2A. Cells activated in type 2 conditions are shown in the upper panels, and those activated in type 1 conditions are shown in the lower panels. On day 7 (left), only CXCR3 was expressed, whereas by day 18 (right), both CXCR3 and CCR5 were expressed. These data are representative of two experiments.
a hapten have been reported in CXCL10-deficient mice (50). It may be relevant in this regard that we found CXCR3 expressed on a subset of human memory B cells both in the tonsil and in peripheral blood (not shown). And in mice we have found that B cell activation induces the expression of functional CXCR3 (49). CXCR3 may play a role in optimizing B cell-Th cell-dendritic cell interactions at the margins, or within germinal centers. Our speculations on possible roles for CXCR3 within lymphoid organs are shown schematically in Fig. 8.

Published work on CXCR3 has focused on its role on differentiated effector/memory cells, particularly on its expression on Th1 cells and the association of CXCR3 and its ligands with type 1 responses in peripheral tissues (11, 12, 14, 15, 19–21). However, other data have cast doubt on the strength of the type 1 bias of CXCR3. For example, using PHA-activated cord blood CD4+ T cells or Ag-activated cells from atopic individuals, CXCR3 was found to be up-regulated equally under both type 1 and type 2 conditions (13). Our in vitro data help reconcile some of these apparently discordant observations. CXCR3 can indeed be up-regulated by activation of naive T cells irrespective of the cytokine environment, and similarly, it can be maintained at high levels on activated cells irrespective of their Th1/Th2 phenotype. Nonetheless, once the cells come to rest, CXCR3 expression is maintained preferentially on Th1 cells, consistent with the strong association of CXCR3 with Th1 cells in peripheral blood, the vast majority of which are no longer activated.

The ease and rapidity with which, in our earlier experiments (8), we found that activation could up-regulate CXCR3 on naive CD4+ T cells, in contrast with other memory/effector chemokine receptors, led us to hypothesize that CXCR3 is expressed early in a program of sequential induction of chemokine receptors during CD4+ T cell differentiation. We investigated this possibility by 1) comparing peripheral blood CD4+ T cells that expressed CXCR3 alone vs those that also expressed CCR5, a receptor found mostly on CD4+ T cells (and as-yet replicated in vitro. Even on these cells, CXCR3 expression may have preceded that of CCR5.

Although we have focused on receptors preferentially expressed on Th1 cells, recent data suggest that there may also be a temporal hierarchy of induction for Th2 cell-associated chemokine receptors such as CCR4 and CCR8 (52). Of additional interest, these studies as well as our own (K. Song, R. L. Rabin, and J. M. Farber, unpublished data) have found that CCR4 is initially induced on cells activated in type 1 and type 2 environments but does not persist on

FIGURE 8. CXCR3 in lymphoid organs. Recruitment of T cells and dendritic cells to the T cell zones from blood or lymphatics is mediated by CCR7 and its ligands CCL21 and CCL19, and recruitment of CD4+ T cells (and B cells) to B cell follicles is mediated through CXCR5 and its ligand CXCL13. Once CD4+ T cells are recruited to these compartments/sites, we propose that, for subsets of cells, CXCR3 and CXCL9 may serve to position activated and/or memory CD4+ T cells and optimize interactions with APC and/or B cells within the T cell zones and at the margins of germinal centers. Activated/memory B cells can also express CXCR3. HEV, High endothelial venules; Ly, lymphatic. A germinal center is represented below: LZ, Light zone; DZ, dark zone; B, B cells. CD4+ T cells are designated as follows: N, naive; M, memory; T, transitional. Chemokine receptors expressed by these cells are written in abbreviated forms: R7 or r7 (for expression at reduced levels), CCR7; XR5, CCR5; XR3, CXCR3. Chemokines mediating recruitment or positioning within tissue are written in italics over the arrows representing the directions of cell movement.
differentiated Th1 cells, a pattern analogous but inverse to that of CXCR3. A variety of models have been proposed for organizing the large number of chemokine receptors expressed on lymphocytes. Our data, along with published studies (7, 51), suggest that an important factor in determining the induction of chemokine receptors on CD4+ T cells is the duration of activation/proliferation, so that receptors are acquired sequentally during differentiation with patterns of expression modified, as pathways of differentiation are modified, by cytokines and other factors. CC-R7 is expressed on naive and central memory cells (7), whereas CCR5 is expressed primarily on effector memory cells (Refs. 7 and 37, and our data). CXCR3 demonstrates a third pattern, as it is induced rapidly after activation of naive cells and persists in the population of Th1 effector cells, including those that express CCR5. And we have shown that this temporal program is associated with a corresponding anatomic pattern, where CXCR3 and its ligands are expressed not only in peripheral inflammation but also within activated lymphoid organs. Together, our data suggest roles for CXCR3 at all stages of differentiation of memory CD4+ T cells and at all sites, both central and peripheral, where these cells act.

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