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Cutting Edge: Induction of Follicular Homing Precedes Effector Th Cell Development[1]

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Transition from naive to Ag-experienced effector/memory CD4$^+$ T cells is initiated during contact with APC in secondary lymphoid tissue. Here, we demonstrate that the CXCR5 is a marker for recently activated memory CD4$^+$ T cells. CXCR5 is rapidly induced during contact with Ag-presenting dendritic cells, well before T cell expansion and effector cell development, and is irreversibly lost on terminally differentiated effector cells. Furthermore, immunization of human volunteers with a recall Ag results in rapid accumulation of Ag-responsive, CXCR5-expressing CD4$^+$ T cells in peripheral blood. Early acquisition of a new migration program enables T zone CD4$^+$ T cells to develop into follicular B helper T cells or, alternatively, into circulating memory CD4$^+$ T cells. Together, CXCR5 unequivocally defines pre-effector memory CD4$^+$ T cells generated during ongoing immune responses. ***The Journal of Immunology*****, 2001, 167: 6082–6086.**

Migration of lymphocytes during maturation, inflammatory responses, and immune surveillance is largely controlled by chemokines and adhesion molecules (1–3). Accordingly, chemokines are grouped into those that control homeostatic cell traffic in lymphoid and extralymphoid tissues, and those that recruit effector cells to sites of inflammation. In secondary lymphoid tissues, recruitment of circulating T cells is mediated by CCR7, which recognizes the two local homeostatic chemokines, secondary lymphoid organ chemokine (SLC,[3] recently designated CCL21) (4) and EBV-induced molecule-1 ligand chemokine (CCL19) (1, 2, 5, 6).

In contrast to these T zone-selective chemokines, the expression of another secondary lymphoid tissue chemokine B cell-attracting chemokine 1 (BCA-1, CXCL13) is restricted to B cell follicles and B cell lymphomas (7–11) and was shown to be a critical regulator of follicular architecture (12, 13). BCA-1 interacts with a single chemokine receptor, termed CXCR5, present on all circulating mature B cells and a minor subset of T cells characterized by their Ag-experienced (CD45RO$^+$) and helper (CD4$^+$) phenotype (7, 9, 14, 15). CXCR5, in analogy to BCA-1, is important for B cell follicle formation in secondary lymphoid tissues (16), and CXCR5$^+$ cells, including T cells generated during primary immune responses and B cells, home to these sites (17). In humans, CXCR5$^+$ T cells are highly enriched in the T zone and B cell follicles of inflamed tonsils (9, 10). Of note, tonsillar CXCR5$^+$ T cells are potent inducers of Ab production during coculture with B cells and, consequently, are referred to as follicular B helper T (T_{FH}) cells (9, 10, 18, 19). The present study identifies CXCR5 as a common and early marker for newly generated memory CD4$^+$ T cells. As such, CXCR5 expression defines a brief and early window in the multistep process leading to Th cell responses and memory formation.

Materials and Methods

Flow cytometry

Ab to CD4 (RPA-T4), CD27 (M-T271), CD45RO (UCHL1), CD45RA (HI100), IFN-γ (25723.22), and IL-4 (3010.211) from BD PharMingen (San Jose, CA) and a rabbit IgG to CXCR5 (9, 11) were used for three-color flow cytometry. Secondary and control Ab: goat-anti rabbit IgG (F-1262; Sigma-Aldrich, St. Louis, MO), mouse IgG1, IgG2a and IgG2b (BD PharMingen), rabbit IgG (Zymed Laboratories, San Francisco, CA). Samples were analyzed on a FACScan (BD PharMingen).

Cell preparation and culture

Isolation of peripheral blood and tonsillar T cells is described (14). Immature dendritic cells (DC) were obtained by culturing CD14$^+$ monocytes with 50 ng/ml IL-4 and 100 ng/ml GM-CSF (PeproTech, London, U.K.) for 5 days. Mature DC1 were obtained by stimulation of immature DC for 8 h with 100 ng/ml LPS; mature DC2 were obtained by culturing immature DC for 2 days with 1 μM PGE_2 or 100 ng/ml LPS (Sigma) (20, 21). Before coculture with T cells (9×10^5 T cells/well), DC (1×10^5 DC/well) were loaded with three superantigens (staphylococcal enterotoxin A, staphylococcal enterotoxin B, and toxic shock syndrome toxin-1; Alexis Biochemicals, San Diego, CA). Alternatively, T cells were stimulated with 5 μg/ml immobilized anti-CD3 Ab (Tr66) or 1 μg/ml PHA. No exogenous IL-2 was added to these cultures.

Proliferation, cytokine production, and chemotaxis

CD4$^+$CD45RO$^+$CXCR5$^+$, CXCR5$^-$ or CD27$^-$, and CD4$^+$CD45RO$^-$ PBL were sorted, and 20,000 cells per well were cultured in complete RPMI 1640 medium containing 500 U/ml human IL-2 on titrated immobilized anti-CD3 Abs (Tr66). Alternatively, sorted CXCR5$^+$ or CXCR5$^-$ fractions of peripheral blood CD4$^+$CD45RO$^+$ T cells from volunteers, before or 6 days after reimmunization with tetanus toxoid (Berna, Bern, Switzerland) were cocultured with sorted, irradiated (45 Gy), autologous or heterologous monocytes (50,000 per well) in medium containing 5% autologous serum in the presence or absence of 5 μg/ml tetanus toxoid. After

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[3] Abbreviations used in this paper: SLC, secondary lymphoid organ chemokine; BCA-1, B cell-attracting chemokine 1; DC, dendritic cell; T_{FH}, follicular B helper T; TREC, TCR rearrangement excision circles; sj, signal joint.

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5 days [^{3}H]thymidine incorporation was measured as described above. Detection of intracellular cytokines was performed as described (9). Cell migration was measured in 48-well chemotaxis chambers (Neuroprobe, Cabin John, MD) as described previously (14).

TCR rearrangement excision circles (TREC) PCR

PCR primers for signal joint (sj)-TREC and amplification procedures were previously described (22). DNA was isolated from 5×10^5 sorted CXCR5$^+$CD4$^+$CD45RO$^+$, CXCR5$^-$CD4$^+$CD45RO$^+$, and CD4$^+$CD45RO$^-$ T cells from PBL or tonsils and from various CD4$^+$ cell lines, using the QIAamp mini kit (Qiagen, Valencia, CA). Template DNA samples were standardized by PCR of genomic glyceraldehydes dehydrogenase and β-actin sequences.

Results and Discussion

Immunization-induced accumulation of Ag-responsive CXCR5$^+$CD4$^+$ T cells in peripheral blood

CXCR5-positive T cells constitute a minor fraction within peripheral blood memory CD4$^+$ T cells (9, 10, 15, 19). To understand the relationship between CXCR5$^+$ expression and memory function, we have studied the proliferation responses of peripheral blood CD4$^+$ T cells to the recall Ag tetanus toxoid. Cells were tested from human volunteers who were vaccinated 8–12 years ago before and 6 days after revaccination. In all volunteers, responses to tetanus toxoid were exclusively detected in CXCR5-negative memory CD4$^+$ T cells, whereas no responses were seen with CXCR5$^+$ memory CD4$^+$ T cells (Fig. 1, *A* and *B*). However, 6 days after reimmunization, marked proliferation responses to tetanus toxoid were now observed in the CXCR5$^+$ memory CD4$^+$ T cells. As expected, both T cell fractions responded well to heterologous monocytes whereas autologous monocytes in the absence of tetanus toxoid did not induce proliferation. Of note, responses to heterologous monocytes were consistently more prominent in CXCR5$^+$ T cells than in CXCR5-negative T cells, both before ($p < 0.002$) and after ($p < 0.02$) immunization with the recall Ag, suggesting that the CXCR5$^+$ cell fractions were enriched for recently activated CD4$^+$ T cells (Fig. 1*B*). These vaccination experiments demonstrate that the pool of circulating CXCR5$^+$ memory CD4$^+$ T cells mirrors recent or ongoing immune activation processes, as opposed to CXCR5-negative CD45RO$^+$CD4$^+$ T cells, representing the pool of "old" memory T cells.

The majority of CXCR5$^+$ T cells in peripheral blood lack prototypic effector functions (Fig. 2*A*), which agrees with previous reports (9, 10, 19). Except for IL-2, which is strongly produced (not shown), peripheral blood CXCR5$^+$ T cells are virtually devoid of cells producing IL-4 (or IL-5, IL-10, and IL-13; data not shown). Considerably more but still minor numbers (<12%) of CXCR5$^+$ T cells produced IFN-γ, suggest that some of these cells have reached early stages in effector T cell differentiation (23). This is in clear contrast to the enhanced cytokine production in CXCR5-negative or fully differentiated (CD27-negative) memory CD4$^+$ T cells (Fig. 2*A*). However, sensitivity of CXCR5$^+$ T cells to anti-CD3-induced proliferation is indistinguishable from the CXCR5-negative and CD27-negative memory fractions, indicating their memory phenotype (Fig. 2*B*). Together, CXCR5 defines peripheral blood memory CD4$^+$ T cells with a nonpolarized phenotype and a recent history of immune activation.

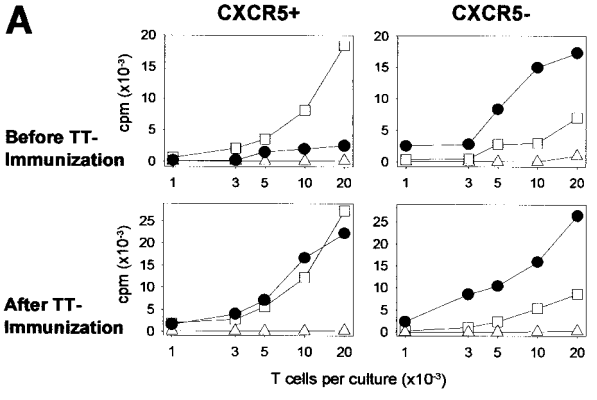

FIGURE 1. In vivo generation of Ag-specific CXCR5$^+$ T cells. Peripheral blood T cells from human volunteers, who were vaccinated against tetanus toxoid 8–12 years ago, were analyzed for reactivity to tetanus toxoid before or 6 days after reimmunization with the recall Ag. *A*, CXCR5$^+$ and CXCR5$^-$ T cells were sorted from peripheral blood memory CD4$^+$ T cells, and increasing numbers were stimulated with autologous monocytes in the presence (●) or absence (△) of tetanus toxoid. Proliferation responses were evaluated by [^{3}H]thymidine incorporation in duplicate cultures. As control, T cells were cocultured in the presence of heterologous monocytes in the absence of tetanus toxoid (□). The results are representative of four independent experiments. *B*, Sorted CXCR5$^+$ and CXCR5$^-$ memory CD4$^+$ T cells (2×10^4 cells per well in duplicates) from four individuals (circles, squares, diamonds, triangles) were analyzed for proliferation in response to autologous monocytes in the presence (+TT) or absence (−TT) of tetanus toxoid or heterologous monocytes in the absence of tetanus toxoid (control). Open and filled symbols refer to T cell isolation before and after boosting of individuals with tetanus toxoid, respectively.

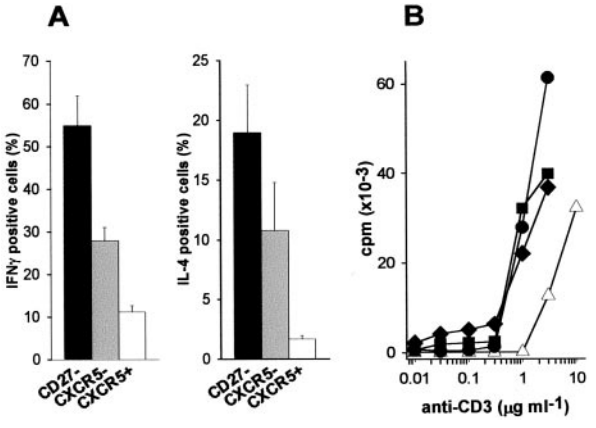

FIGURE 2. Enhanced Ag-sensitivity but nonpolarized phenotype of peripheral blood CXCR5$^+$ memory CD4$^+$ T cells. *A*, Purified naive (CD45RO$^-$) or memory (CD45RO$^+$) CD4$^+$ T cells from peripheral blood were stimulated with PMA and ionomycin and analyzed for intracellular production of cytokines by three-color flow cytometry. Gates were set on CD27$^-$ (filled; $n = 3$), CXCR5$^-$ (gray; $n = 11$), and CXCR5$^+$ (open; $n = 11$) CD4$^+$ T cells; mean values ± SEM are shown. *B*, Sorted peripheral blood CD27$^-$ (■), CXCR5$^-$ (♦), CXCR5$^+$ (●), and naive (△) CD4$^+$ T cells were examined for proliferation ([^{3}H]thymidine incorporation in duplicate cultures) in response to increasing concentrations of plate-bound anti-CD3 Abs.

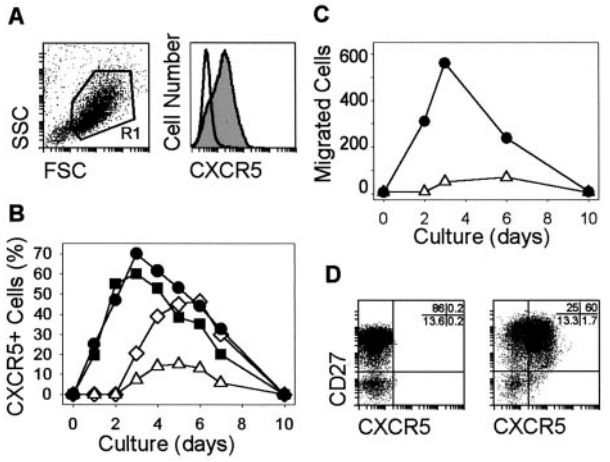

FIGURE 3. Rapid and transient induction of CXCR5 in human peripheral blood $CD4^+$ T cells. *A*, CXCR5 expression in naive $CD4^+$ T cell-derived blasts after stimulation for 3 days with superantigen-loaded LPS-treated DC1 (filled histogram). Open histogram represents control staining with rabbit IgG. *B*, Kinetics of CXCR5 expression in naive $CD4^+$ T cells after stimulation with superantigen-loaded LPS-treated DC1 (●), PGE_2-treated DC2 (■), PHA (◇), or immobilized anti-CD3 Abs (△). Data are representative of five independent experiments. *C*, CXCR5 expression correlates with chemotactic migration to BCA-1. Naive $CD4^+$ T cells were stimulated with superantigen-loaded LPS-treated DC1 (●) or immobilized anti-CD3 Abs (△) and analyzed for migration responses to 1 μM BCA-1 (migrated cells per five high power fields). *D*, Induction of CXCR5 expression in $CD27^+$ vs $CD27^-$ peripheral blood memory $CD4^+$ T cells. $CXCR5^+$ T cell-depleted memory $CD4^+$ T cells were analyzed for CD27 and CXCR5 expression before (*left*) and after stimulation for 3 days with superantigen-loaded LPS-treated DC1 (*right*). One representative of three independent experiments.

Rapid and transient induction of CXCR5 in $CD4^+$ T cells during Ag recognition

The presence of Ag-responsive CXCR5-expressing T cells in peripheral blood during vaccination suggests that this chemokine receptor is induced during initiation of immune responses. Therefore, we examined the requirements for induction of CXCR5 in human naive $CD4^+$ T cells, which completely lack this chemokine receptor. Peripheral blood naive ($CD45RO^-$) $CD4^+$ T cells were stimulated with autologous superantigen-loaded monocyte-derived DC, PHA, or immobilized anti-CD3 Abs. T cell blasts, identified by enhanced forward and side scatter fluorescence (Fig. 3*A*), acquired uniform expression of activation (CD69), costimulation (inducible costimulator) and memory (CD45RO) markers (data not shown). Superantigen-loaded DC rapidly induced cell surface CXCR5 expression, with maximal numbers obtained by day 3 of culture (Fig. 3*B*), whereas DC in the absence of superantigen had no effect (not shown). The induction of CXCR5 was not dependent on the type of DC used. Type 1 DC (DC1), obtained by maturation in the presence of LPS or type 2 DC (DC2), generated by treatment with PGE_2 (20) or, alternatively, by exhaustion of LPS-cultured DC1 (21), induced CXCR5 expression with the same kinetics and efficacies. These results indicate that induction of CXCR5 is not controlled by T cell polarization events, leading to cytokine producing effector cells. When naive cells were stimulated with PHA or anti-CD3 Abs, CXCR5 expression was markedly reduced, both in terms of kinetics and maximal numbers of chemokine receptor-positive cells (Fig. 3*B*). In common to all culture conditions, CXCR5 expression was transient. In DC-treated cells the decline of cell surface CXCR5 was observed at early stages of T cell proliferation (more than day 3 of culture), whereas in PHA- or anti-CD3-treated cells the decrease was delayed by several days. Functional integrity of newly generated CXCR5 was verified by chemotactic migration to BCA-1, and efficacy of these responses paralleled CXCR5-positivity in activated T cells (Fig. 3*C*). We conclude that $CD4^+$ T cells that are engaged in early stages of primary immune responses are typified by CXCR5 and acquire a new migration profile.

In peripheral blood, CXCR5 characterizes a minor subset of memory ($CD45RO^+$) $CD4^+$ T cells (9, 10, 19), but we find that most of CXCR5-negative memory $CD4^+$ T cells retained the capacity to transiently express CXCR5. Again, highest numbers of $CXCR5^+$ cells were obtained by stimulation with superantigen-loaded DC (data not shown). By contrast to naive T cells (Fig. 3*B*), there was no delay in PHA- or anti-CD3-mediated induction of CXCR5 expression, reflecting the lower threshold level for memory T cell activation (24–26). CXCR5 is not present on terminally differentiated effector cells in peripheral blood, distinguished by lack of CD27 (27) and homing preferences for inflammatory sites (1–3). By contrast to $CD27^+$ memory $CD4^+$ T cells, these cells have irreversibly lost the capacity to up-regulate CXCR5 (Fig. 3*D*). Moreover, cultured $CD4^+$ T cell lines or clones with defined cytokine profiles (T_H0, T_H1, T_H2) lack CXCR5 and, like blood CD27-negative memory $CD4^+$ T cells, no longer express CXCR5 during restimulation (data not shown). Therefore, induction of

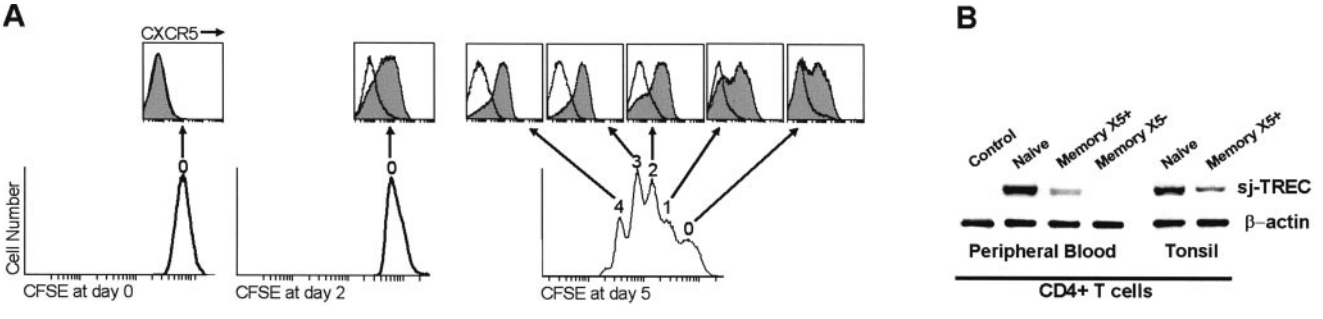

FIGURE 4. CXCR5 marks recently activated $CD4^+$ T cells with a short proliferative history. *A*, Induction of CXCR5 expression in peripheral blood naive $CD4^+$ T cells occurs early during stimulation with superantigen-loaded LPS-treated DC1 and precedes entry into cell division. T cells were labeled with CFSE and cocultured with DC at a ratio of 9:1. After 0, 2, and 5 days, cell populations with zero to four cell divisions were analyzed for CXCR5 expression. Data are representative of three independent experiments. *B*, $CXCR5^+$ memory $CD4^+$ T cells from blood and tonsils are enriched for cells with a short proliferative history. sj-TREC DNA was readily detected by PCR amplification with DNA from peripheral blood and tonsillar $CXCR5^+$ memory $CD4^+$ T cells (Memory X5+). Control, DNA from a $CD4^+$ T cell line; β-actin, genomic β-actin DNA. Data are representative of three independent experiments.

CXCR5 expression is restricted to $CD27^+$ memory T cells and excludes terminally differentiated effector $CD4^+$ T cells, which have lost secondary lymphoid tissue homing capabilities.

Modulation of cell surface CXCR5 during $CD4^+$ T cell proliferation

Rapid CXCR5 expression in naive and memory $CD4^+$ T cells suggests that this event precedes the onset of T cell proliferation and differentiation. This was examined by correlating entry into cell cycle, assessed by fluorescence reduction in CFSE-labeled naive $CD4^+$ T cells (26), with induction of CXCR5 expression during stimulation with DC. After 2 days, the majority (74%) of T cells has already acquired cell surface CXCR5 although they have not yet divided (Fig. 4*A*). By day 5 of culture, 66% of $CD4^+$ T cells went through two to four cell divisions and these cell populations contained 75–80% $CXCR5^+$ cells, whereas CXCR5 expression in the minor fraction of nondivided cells was reduced to <50%. Of note, highest levels of CXCR5 expression correlated with those fractions, which were first to enter cell cycle, and CXCR5-positivity was maintained over the initial two to four cell divisions. Further cell culture (>5 days) resulted in a decrease of cell surface CXCR5 in all cell fractions (data not shown). These studies demonstrate that induction of CXCR5 expression occurs early during T cell activation and does not require cell cycle-dependent epigenetic modifications, which clearly contrasts initiation of $T_H1/2$-type cytokine production (23, 28). Furthermore, the ability to produce IFN-γ is installed during the first few divisions, which agrees with the maintenance of cell surface CXCR5 during early stages of T cell proliferation and the observed occurrence IFN-γ-producing $CXCR5^+$ T cells in peripheral blood (Fig. 2*A*). Reduction in cell surface CXCR5 may have resulted from loss of T cell contact with DC, and/or extended T cell proliferation.

Cells with rapid and transient expression of CXCR5 were further studied by examining the content of TREC (22). TCR gene rearrangement during thymocyte development results in the generation of DNA excision circles, which are "diluted out" during subsequent cell divisions. Analysis of TREC by PCR is frequently used to identify thymic emigrant cells in peripheral blood, including mature naive T cells (22). sj-TREC were readily detected in DNA extracted from naive $CD4^+$ T cells independent of their origin (peripheral blood, tonsils), but were not found in DNA from CXCR5-negative memory $CD4^+$ T cells (Fig. 4*B*). As expected, DNA from various T cell lines lacks detectable levels of sj-TREC. In contrast, sj-TREC were readily amplified with DNA from sorted CXCR5-positive memory $CD4^+$ T cells from peripheral blood or tonsils. Possibly the observed sj-TREC content in $CXCR5^+$ memory $CD4^+$ T cells originated mainly from naive $CD4^+$ T cell-derived $CXCR5^+$ cells, characterized by limited proliferation. Together, these studies demonstrate that induction of CXCR5 expression precedes T cell proliferation and is maintained on the cell surface only for a limited number of cell divisions.

We propose that $CXCR5^+$ Th cells in secondary lymphoid tissues possess three options for further development. First, by means of CXCR5 expression, activated Th cells in the T zone may be recruited into B cell follicles, which selectively express BCA-1 (7–10, 19). Possibly, this feature ensures that humoral immune responses can rapidly benefit from an unrestricted range of Ag-priming information generated in the T zone. Because most $CXCR5^+$ T cells in blood or tonsils are nonpolarized (9, 10, 19), development into effective T_{FH} cells may occur locally during contact with B cells and/or follicular stromal cells. Second, $CXCR5^+$ Th cells may reside in the T zone long enough to complete the full proliferation and differentiation program, which results in the generation of effector cells with characteristic cytokine profiles. This process is accompanied by a switch in migration properties to provide immediate immune defensive functions at inflammatory sites (1–3). Third, $CXCR5^+$ Th cells have the option to exit secondary lymphoid tissues and to form the pool of "recent" memory $CD4^+$ T cells in peripheral blood, as shown here during recall responses in tetanus toxoid vaccinated individuals. The majority of tonsillar $CXCR5^+$ memory $CD4^+$ T cells shows greatly reduced responsiveness to T zone chemokines (SLC and EBV-induced molecule-1 ligand chemokine) and, therefore, is no longer retained in this microenvironment (9, 10). Importantly, exit of memory $CD4^+$ T cells from secondary lymphoid tissues shortly after initiation of T cell activation ensures maintenance of the diversity in Ag selectivity by evading local apoptotic processes. Because BCA-1 is also present on follicular venules (but not on interfollicular HEV) (9), newly generated $CXCR5^+$ memory $CD4^+$ T cells in peripheral blood may be targeted directly to B cell follicles of other secondary lymphoid tissues for potential propagation of the humoral immune response. Alternatively, entry into peripheral blood may preserve their capability for development into effector Th cells during a subsequent recall response (24, 25). Clearly, this places the formation of nonpolarized CXCR5-expressing memory Th cells at an early stage of immune responses whereas fully differentiated memory Th cells ($T_H1/2$ cells) may be generated later, possibly during the phase of effector T cell exhaustion (25, 26). Old memory Th cells lack CXCR5, as evidenced by our tetanus toxoid vaccination experiment. T cell proliferation results in loss of cell surface CXCR5 and, therefore, we propose that old nonpolarized memory Th cells are derived (at least in part) from recent $CXCR5^+$ memory Th cells, due to base level proliferation, which is required for long-term maintenance of T cell memory (24–26, 29). During recall responses, these old memory Th cells rapidly reacquire CXCR5 for further development into T_{FH}, cytokine-producing effector and recent ($CXCR5^+$) memory Th cells.

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