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The Chemokine CXCL13/BLC directs lymphocyte homing and immune function

by

K. Mark Ansel

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

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of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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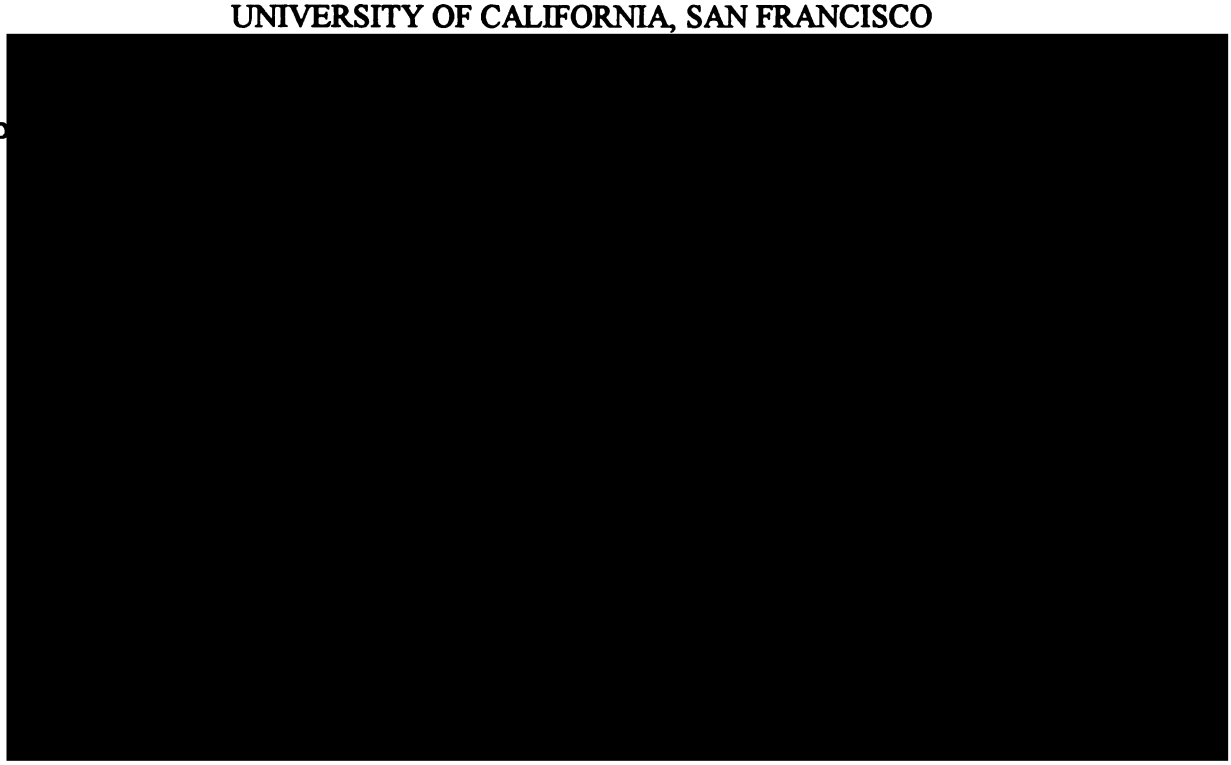

This dissertation is dedicated to my parents,

Rick and Sandy Ansel,

who have loved and supported me through all that I have chosen to do,

and who made me believe that I can do anything.

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Contribution to presented work

Chapter 2 of this dissertation is a reprint of the material as it appears in *Nature,* Ansel, K.M., V.N. Ngo, P.L. Hyman, S.A. Luther, R. Förster, J.D. Sedgwick, J.L. Browning, M. Lipp, and J.G. Cyster, (2000) 406:309-14. This work was a collaborative effort between Vu Ngo and myself, with smaller contributions and reagents provided by the other listed coauthors. I generated the CXCL13-deficient mice and contributed data to all of the figures excepts figures 3 and 4. I designed or helped to design the experiments described in figures 1, 2, 5, and Table 1, and helped to write the manuscript. Chapter 3 of this dissertation is a reprint of the material as it appears in *The Journal of Experimental Medicine,* Ansel K.M., L.J. McHeyzer-Williams, V.N. Ngo, M.G. McHeyzer-Williams, and J.G. Cyster, (1999) 190:1123-34. I helped to write the manuscript and designed and executed all of these experiments except those presented in figures 2, parts E-F and all of Figure 3. All of the work in Chapter 5 is the result of experiments designed and executed by myself, with the help of Ruth Harris who generated and cared for the parabiotic mice used in figure 6. Appendix 1 of this dissertation is a reprint of the material as it appears in *Immunological Reviews,* Cyster, J.G., K.M. Ansel, K. Reif, E.H. Ekland, P.L. Hyman, H.L. Tang, S.A. Luther, and V.N. Ngo, (2000) 176:181-193. I helped to revise the manuscript and contributed data to figure 4. Appendix 2 of this dissertation is a reprint of the material as it appears in *Current Opinions in Immunology,* Ansel, K.M. and J.G. Cyster (2001) 13:172-9. I researched and wrote the sections on "stem cell homing and B cell lymphopoiesis" and "chemokines in lymphoid organogenesis" for this review and

created all of the figures and table 1. Appendix 3 of this dissertation is a reprint of the material as it appears in *Current Topics in Microbiology and Immunology,* Cyster, J.G., V.N. Ngo, E.H. Ekland, M.D. Gunn, J.D. Sedgwick, and K.M. Ansel, (1999) 246:87-92. I designed and executed the experiments presented in figures 1 and 2 and helped to prepare the manuscript for publication. Appendix 4 of this dissertation is a reprint of the material as it appears in *Nature,* Gunn, M.D., V.N. Ngo, K.M. Ansel, E.H. Ekland, J.G. Cyster, and L.T. Williams, (1998) 391:799-803. I contributed data to figures 3 and 4 and helped prepare the figures for publication. Further acknowledgements of individuals and organizations are included at the end of chapters 2, 3, and 4, and appendices 1-4.

The chemokine CXCL13/BLC directs lymphocyte homing and immune function

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K. Mark Ansel

Secondary lymphoid organs have evolved to facilitate immune surveillance and to orchestrate the cellular interactions that ensue when an invading pathogen is recognized. Within these tissues, recirculatory B and T cells segregate into distinct microenvironments containing specialized stromal and accessory cells that promote their immune function: T cells home to central T zones, while B cells migrate into the adjacent follicles. In recent years, chemokines have emerged as key regulators of lymphocyte trafficking. In the studies described herein, I focussed on the role of CXCL13/BLC, a highly efficacious B lymphocyte chemoattractant made by follicular stromal cells.

Using newly generated CXCL13-deficient mice, I demonstrated that CXCL13 is essential for B cell homing to follicles in all secondary lymphoid organs. CXCL13-deficiency also disrupts follicle homeostasis and B cell expression of lymphotoxin (LT)α1β2, a cytokine that is required for maintenance of the CXCL13-producing follicular stroma. This positive feedback loop is overridden in germinal centers, with high levels of LTα1β2 expressed in a

CXCL13-independent manner. CXCL13-deficient mice also lack most lymph nodes and Peyer's patches, suggesting that the mechanisms that control secondary lymphoid organ homeostasis may also operate in their development. During T-dependent antibody responses, antigen-specific T cells enter follicles and provide signals necessary for germinal center formation. I provided evidence that T cell homing to follicles is controlled by the acquisition of responsiveness to CXCL13 and a simultaneous decrease in responsiveness to chemokines produced in T zones. Finally, I characterized the homing of B1 cells, specialized B cells that play a prominent role in natural antibody production and body cavity immunity. B1 cells are selectively recruited to the peritoneal and pleural cavities in a CXCL13-dependent manner, and both natural antibody production and T-independent responses to intraperitoneally injected streptococcal antigens are defective in the absence of CXCL13. Taken together, these findings establish CXCL13 as a central regulator of B cell homing and immune function.

Table of Contents

List of Tables

List of Figures

List of Figures *(continued)*

List of Figures *(continued)*

List of Figures *(continued)*

Chapter 1

Introduction

Immune surveillance and the role of secondary lymphoid organs

The challenge of immune surveillance is an important one – rapid and efficient encounter of antigen-specific lymphocytes with invading pathogens is crucial for effective host defense. Lymphocytes bearing a vast repertoire of antigen receptor specificities are generated in primary lymphoid organs. While the diversity of this repertoire confers the ability to respond to an equally diverse array of foreign antigens, it also creates a problem for the immune system: The lymphocytes needed to respond to a particular pathogen are very rare. Mature B and T cells recirculate via blood and lymph, patrolling for foreign antigens that they can recognize and attack (1, 2). Secondary lymphoid organs, which include the spleen, lymph nodes, tonsils and Peyer's patches, play a critical role in facilitating immune surveillance, acting as filters that actively concentrate antigens for sampling by the large number of recirculating naïve lymphocytes that continuously migrate to and through them. Accordingly, they are the predominant sites of the initiation of immune responses. The spleen is especially important for responses to bloodborne antigens, while tonsils and Peyer's patches are associated with specialized epithelial cells that sample antigens from the throat and small intestine, respectively. Lymph nodes are found throughout the body, and are an integral part of lymph drainage from both the skin and visceral organs, filtering the lymph fluid before it is returned to the bloodstream via the thoracic duct.

All secondary lymphoid organs share a common basic architecture, with T and B cells segregated into central T zones and the adjacent B cell follicles

(Figure 1). These specialized microenvironments contain stromal cells and other accessory cells that promote antigen encounter and regulate the activation of their respective lymphocyte populations. In addition to T cells, a large number of dendritic cells are found in T zones (3). These sentinels of the immune system capture and process antigens for presentation to T cells in conjunction with costimulatory molecules and cytokines that enhance T cell activation and differentiation (4). As they mature, dendritic cells that capture antigens in peripheral tissues acquire the ability to home to T zones of secondary lymphoid organs, the site where they are most likely to encounter naïve T cells specific for the antigens they are presenting (3, 4). In lymph nodes and Peyer's patches, T zones also contain a large number of high endothelial venules (HEV), specialized vessels that serve as the primary means of lymphocyte entry to these organs. Transendothelial migration of lymphocytes is a multistep process involving selectin-mediated tethering and rolling, activation of integrin-dependent firm adhesion, and finally diapedesis between endothelial cells (1, 5). T zones are also centrally located in the splenic white pulp cords, forming in association with a central arteriole. However, lymphocytes do not enter splenic T zones directly from the central arteriole, instead traversing from the erythrocyte-rich red pulp to the white pulp via bridging channels abutting T zones.

B cells enter secondary lymphoid organs in the same manner as T cells, but proceed through or along the boundary of the T zones into the adjacent follicles. Follicles are polarized structures that contain, in addition to B cells, a heterogeneous population of stromal cells, including follicular dendritic cells

(FDC). FDC do not internalize and process antigens, but can capture and hold them on their surface for months, possibly years in the form of immune complexes of intact antigens in association with antibodies and/or complement C3 and C4 fragments (6, 7). To this end, FDC express immunoglobulin Fc receptors and high levels of the complement receptors CD21/CR2 and CD35/CR1, the latter serving as an appropriate marker for the identification of FDC (8). Both FDC and T zone stromal cells are radiation-resistant and express molecules consistent with classification as non-hematopoietic fibroblasts. Readers interested in a more thorough examination of the development and function of FDC are directed to Appendix 1. In B cells, CD21 is expressed in association with the costimulatory signaling molecule CD19 (9, 10), and antigens covalently linked to complement fragments are 1000 to 10000 times more potent than antigen alone in initiating immune responses (11). Thus follicles, like T zones, concentrate antigens in an appropriate immunogenic form for the lymphocytes that home there, increasing the efficiency of immune surveillance.

Secondary lymphoid organs are also important for orchestrating cell-cell interactions during immune responses. Antibody responses against most protein antigens require T cell help, which in turn depends on physical interaction between antigen specific B and T cells. Upon antigen encounter, B and T cells change their homing pattern within secondary lymphoid organs ((12) and see Figure 2). Activated B cells move to the boundary between follicles and T zones (13-15). In the absence of T cell help (e.g. in the case of autoreactive B cells) antigen-specific B cells are eliminated in about three days (14, 16). However,

activated $CD4^+$ T cells also become enriched at the T-B boundary, juxtaposing antigen-specific T and B cells (17, 18). If successful T-B interaction occurs, some B cells differentiate into antibody-producing plasma cells that form foci in medullary cords in lymph nodes and in bridging channels and the red pulp of the spleen (19-21). Other B cells and some T cells enter follicles and form germinal centers (22, 23). Follicles containing germinal centers are called secondary follicles and develop a higher density of FDC than primary follicles that lack a germinal center (6). Germinal centers are the site of oligoclonal B cell expansion, immunoglobulin gene hypermutation, and antibody affinity maturation (24). $CD4^+$ T cells are required for germinal center function, although abortive germinal centers can be transiently induced in some T-independent systems (25). Memory B cells and most of the long-lived plasma cells that home to the bone marrow emerge from germinal centers (26). In general, both effector and memory T and B cells modulate their expression of homing molecules, leading to a decreased propensity to enter lymph nodes, and an increased presence in peripheral tissues and sites of inflammation (27-29).

Molecular determinants of lymphocyte homing and secondary lymphoid organ homeostasis

It has long been known that lymphocytes have an intrinsic ability to migrate to their respective microenvironments within secondary lymphoid organs, but little was known about the molecular guidance of lymphocyte homing. In recent years, however, a family of small chemotactic cytokines (chemokines)

have emerged as central regulators of cell migration in the immune system (2, 30, 31). The chemokine family is classified into four subfamilies according to the number and spacing of conserved cysteine residues, which are adjacent in CC chemokines and separated by one amino acid in chemokines of the CXC family. To date, the CX_3C and XC families are represented by only one member each. The genomic organization of the chemokine genes suggests considerable genomic instability with frequent duplications, and this may contribute to the prevalence of redundancy in chemokine function and receptor usage (32, 33). A systematic nomenclature system has been proposed for chemokines (34). Though this attempt to standardize chemokine nomenclature deserves support, both the common and systematic names will be found within this dissertation.

Chemokine receptors are members of the seven transmembrane-spanning G protein-coupled receptor family. They couple chiefly to $G\alpha_i$, as evidenced by the pertussis toxin sensitivity of their downstream signaling and physiological effects on chemokine-treated cells (Figure 3). Chemokine binding to chemokine receptors stimulates the release of intracellular calcium stores and activation of PI3K, and in some cases activation of the MAPK, NFκB, and JAK/STAT pathways has been observed (reviewed in (35)). Most importantly for these studies, cells expressing an appropriate chemokine receptor are able to migrate into a gradient of increasing chemokine concentration (36). This directional migration is termed chemotaxis. Signaling via some chemokine receptors leads to a rapid activation of adhesion molecules of the integrin family (37, 38). This effect is critical for chemokine induction of leukocyte firm adhesion

in transendothelial migration, and is likely to play an important role in the migration of lymphocytes within tissues as well (1, 5).

Chemokines have been classified into two groups according to their function in the immune system. Inflammatory chemokines are induced at sites of inflammation and recruit neutrophils, macrophages, and/or activated lymphocytes, whereas lymphoid chemokines are constitutively expressed in secondary lymphoid organs and can attract naïve lymphocytes. Although the distinction between inflammatory and lymphoid chemokines is sometimes blurred, with some induced chemokines attracting naïve lymphocytes and some constitutively expressed chemokines being expressed at sites of inflammation, this classification highlights the unique role that a small number of lymphoid chemokines plays in lymphocyte homing and secondary lymphoid organ homeostasis (2). Four chemokines in particular have been the subject of many recent studies: CXCL12/SDF-1α and its receptor, CXCR4; CCL19/ELC and CCL21/SLC and their shared receptor, CCR7; and the major subject of my dissertation research, CXCL13/BLC and its receptor CXCR5.

CXCL12 was originally cloned as a pre-B cell growth-stimulatory factor produced by bone marrow stromal cells (39). Most hematopoietic cells express CXCR4, though expression of both CXCL12 and CXCR4 are not restricted to the immune system (40). Mice lacking CXCL12 or CXCR4 have identical phenotypes, with cardiac, cerebellar, vascular, and hematopoietic defects, and both suffer perinatal lethality (41-43). CXCR4 and CXCL12 therefore appear to act as a monogamous receptor and ligand pair. Adoptively transferred $CXCR4^{-/-}$

fetal liver cells perform poorly in long-term hematopoietic reconstitution of lethally irradiated recipients, with B cells and myeloid precursors prematurely escaping the bone marrow and appearing in the circulation (44). A more thorough discussion of the role of CXCL12 and CXCR4 in hematopoiesis can be found in Appendix 2. Recently, it has been shown that CXCR4 is required for plasma cell homing to the bone marrow, and $CXCR4^{-/-}$ plasma cell localization within secondary lymphoid organs is also altered (45). Consistent with a role in plasma cell migration, CXCL12 is constitutively expressed by bone marrow stromal cells (40) and in the red pulp of the spleen and medullary cords in lymph nodes (45). Although CXCR4 is expressed in mature B and T cells, its role in these cells has been more difficult to ascertain, and $CXCR4^{-/-}$ fetal liver chimeras have intact T zones and follicles (44).

Recent studies have established that CCR7 plays a critical role in directing T cell and dendritic cell homing to T zones in secondary lymphoid organs. Both CCL19 and CCL21 are constitutively expressed by T zone stromal cells, but not by FDC or other stromal cells outside T zones (30, 46). CCL19 is also expressed at low levels by a subset of dendritic cells (47), and lymphatic vessels and HEV express CCL21 (48). Naïve T and B cells both express CCR7 and respond to CCL19 and CCL21, though the response of T cells is greater. $CCR7^{-/-}$ mice and a natural mutant lacking all CCL19 and CCL21 expression by T zone stroma and HEV (*plt/plt* mice) have drastically reduced numbers of lymph node T cells and dendritic cells (46, 49-51). Splenic T cell zones of these mice are also small and hypocellular, with a particular paucity of naïve T cells (49, 51). In contrast, B cell

homing to lymph nodes and follicular organization are intact in the absence of CCR7 or its ligands (49, 52). Compared to wildtype T cells, adoptively transferred $CCR7^{-/-}$ T cells home very inefficiently to lymph nodes and splenic T zones (49). Similarly, wildtype T cells home poorly in *plt/plt* mice (52). Although the efficiency of $CCR7^{-/-}$ B cell entry into splenic white pulp cords may be slightly impaired, B cell homing to follicles is not directed by CCR7 (49).

Instead, a third chemokine receptor expressed by naïve B cells, CXCR5, fulfills this function. CXCR5 was cloned from a Burkitt's lymphoma cell line, and was originally termed Burkitt's lymphoma receptor (BLR)-1 (53). CXCR5 is not expressed by early B cells precursors, except for a very low level of expression on pro-B cells ((54) and Appendix 3). Bone marrow immature B cells begin to express CXCR5, and expression increases in transitional B cells as they mature in the periphery ((54) and Appendix 3). With the notable exception of differentiated plasma cells, all mature B cells express CXCR5, including splenic marginal zone B cells and peritoneal B1 cells ((54) and Appendix 3). Outside of the B cell lineage, CXCR5 has been detected on a subset of T cells with a "memory" surface phenotype (55), and may also be expressed by a small population of follicle-homing dendritic cells (56). B cells fail to form polarized follicles in $CXCR5^{-/-}$ mice, instead forming a loosely organized ring around the central T zones (57). In adoptive transfer experiments, $CXCR5^{-/-}$ B cells failed to home to follicles in the spleen and Peyer's patches (57), and subsequent studies revealed a similar defect in homing to lymph node follicles (Chapter 2).

The only known ligand for CXCR5, CXCL13/BLC, was cloned just prior to the initiation of my thesis research ((58), and Appendix 4). Consistent with a role in directing B cell homing in secondary lymphoid organs, CXCL13 is constitutively expressed by stromal cells in lymphoid follicles, but not in T zones (Appendix 4). We and others (58) showed that primary B cells and cell lines transfected with CXCR5, but not other known chemokine receptors, flux calcium and chemotax in response to CXCL13 *in vitro* (Appendix 4). CXCL13 also stimulates CXCR5 desensitization and internalization from the plasma membrane (unpub. obs.). Of the known B cell chemoattractants, CXCL13 is the most efficacious, with up to eighty percent of B cells migrating in *in vitro* chemotaxis assays. The studies described herein highlight the importance of CXCL13 in directing lymphocyte homing as well as in the development and homeostasis of secondary lymphoid organs.

Three members of the tumor necrosis factor (TNF) family also play critical roles in lymphoid organ development and homeostasis (reviewed in (59)). These cytokines bind their receptors as homo- or heterotrimers, with some receptors recognizing more than one combination of ligands. TNF and lymphotoxin (LT)α homotrimers share the receptor TNFR1, and LTα can also associate with membrane-bound LTβ, forming LTα1β2 heterotrimers with the ability to bind a second receptor, LTβR. Follicular organization is impaired in mice lacking TNF, with B cells ringing T zones but failing to form polarized structures containing FDC (59). A similar phenotype is observed in mice deficient for TNFR1 (59). The disorganization of splenic architecture is most severe in mice deficient for LTα,

LTβ, or LTβR, with T and B cells failing to segregate into well-defined zones, and the absence of both FDC and T zone stromal markers ((59, 60), and unpub. obs.). Mixed bone marrow chimera experiments revealed that B cells, but not T cells, are a necessary source of lymphotoxin for maintenance of T-B organization and the FDC network (59). Similar defects are manifest in mice lacking signaling molecules in the LTβR pathway. Strikingly, expression of CXCL13 and CCL21 are decreased to less than 5% of wildtype levels in the spleen of lymphotoxin-deficient mice (61). CCL19 expression is also reduced, but CXCL12 expression is lymphotoxin-independent (61). Importantly, blocking LTβR ligation in adult mice by treating with an LTβR-Fc fusion protein for several days also results in decreased chemokine expression (61), loss of FDC markers, and disrupted T-B organization in spleen and lymph nodes (62). Taken together, these data establish a critical role for lymphotoxin in secondary lymphoid organ homeostasis (59).

Secondary lymphoid organ development

In addition to these defects in secondary lymphoid organ homeostasis, lymphotoxin-deficient mice lack Peyer's patches and nearly all lymph nodes ($LT\beta^{-/-}$ mice and less than 10% of $LT\alpha^{-/-}$ mice develop a single large mesenteric lymph node) (59). Unexpectedly, $CXCR5^{-/-}$ mice were also reported to lack Peyer's patches and some lymph nodes, suggesting that the mechanisms that maintain lymphoid organ homeostasis also operate in their development (57). Significant progress has been made in the elucidation of these mechanisms, with

most of the work focussed on the development of Peyer's patches. Peyer's patch organogenesis has been dissected into three distinctive steps: *i)* Interleukin-7 receptor (IL7R)α-initiated LTα1β2-dependent induction of the adhesion molecules, VCAM-1 and ICAM-1 on intestinal mesenchymal cells; *ii)* coalescence of scattered IL7Rα$^+$ cells at focal sites of VCAM/ICAM expression; and *iii)* colonization of the Peyer's patch anlagen by B cells and T cells (63). The IL7Rα$^+$ cells that first colonize Peyer's patch anlagen are CD45$^+$CD4$^{+/-}$CD3$^-$ and express homing receptors including CXCR5 and the integrin $\alpha_4\beta_7$ (64). Analysis of developing lymph nodes in fetal and newborn mice revealed the presence of a remarkably similar cell type (65). Furthermore, these cells fail to develop in mutant mice lacking the transcriptional repressor Id2 or the orphan hormone receptor RORγ, and these animals lack all lymph nodes and Peyer's patches (66, 67). In contrast, lymph node development is intact in mice lacking B and T cells. Therefore, it is likely that organogenesis of Peyer's patches and lymph nodes proceeds via similar mechanisms induced by common (or closely related) CXCR5-expressing lymphoid tissue inducing cells (LTICs).

Underscoring the critical role of secondary lymphoid organs in adaptive immunity, mice lacking lymphotoxin have diminished antibody responses to T-dependent antigens and defective germinal center formation (59). Like primary follicles in these mice, the rare germinal centers that do form in the mesenteric lymph nodes of LTβ$^{-/-}$ mice contain no FDC (68). CCR7$^{-/-}$ and *plt/plt* mutants exhibit delayed T-dependent antibody responses and impaired delayed type hypersensitivity despite normal development of lymphoid organs (69). Therefore,

lymphocyte homing to specific microenvironments within secondary lymphoid organs is also important for immune function. In particular, the delay in T-dependent antibody responses suggests impaired immune surveillance.

B1 cells: Lymphocytes with distinct homing properties and specialized immune function

B1 cells are present at a low frequency in the spleen, but are the major population of lymphocytes in body cavities, including the peritoneal, pleural, and pericardial cavities. In addition to this unique tissue distribution, B1 cells are distinguished from conventional recirculating (B2) B cells by their capacity for self renewal and restricted antigen receptor repertoire (70, 71). The differentiation of B cell precursors is influenced by antigen receptor specificity, with certain immunoglobulin gene rearrangements restricted to expression in B1 cells (71). One well-characterized example of a B1-associated antigen receptor is the T15 idiotype-containing germline-encoded immunoglobulin specific for the bacterial hapten, phosphorylcholine (PC) (72, 73). Generally speaking, immunoglobulins gene rearrangements without junctional nucleotide additions favor the production of B1 cells of the $CD5^+$ B1a subset (72). Therefore, B1a cells develop primarily from fetal hematopoietic precursors that do not express terminal deoxynucleotide transferase (TdT) and TdT^+ adult hematopoietic precursors are unable to fully reconstitute the B1a compartment of lethally irradiated mice (70). The $CD5^-$ B1b cells, which are otherwise phenotypically very similar to B1a cells, have a

somewhat more diverse antigen receptor repertoire, and can be generated from adult hematopoietic precursors (70, 71).

B1 cells are a minor subset of B cells, but a major source of natural antibodies (74, 75). These low affinity IgM antibodies are present in the serum in the absence of apparent immunization, and can therefore bind their cognate antigens and induce complement fixation immediately upon antigenic challenge (76). Mice deficient in natural antibodies are susceptible to a variety of pathogens (77-80), and an especially prominent role has been established for PC-specific natural antibodies produced by B1a cells in protection against extracellular bacteria (72, 81). In addition to this role in innate immunity, B1 cells can participate in T-independent antibody responses in the spleen and peritoneum (82). B1 cells are also associated with autoimmunity, and studies using autoreactive immunoglobulin-transgenic mice support a model in which localization within body cavities protects at least some autoreactive B cells from deletion induced by contact with peripheral autoantigens (83). Despite the importance of the B1 cell population in immune function and dysfunction, and speculation about the importance of their unique tissue distribution, little is known about how B1 cells enter the body cavities, or what factors guide their homing.

Overview of thesis work

The goal of these studies was to elucidate the mechanisms that control lymphocyte homing and secondary lymphoid organ development and homeostasis, with particular interest in B cells and lymphoid follicles. The

identification of CXCL13 as a B lymphocyte chemoattractant and ligand for CXCR5 launched a series of experiments aimed at dissecting the physiological activity of this chemokine. CXCL13$^{-/-}$ mice were generated by gene targeting and were found to share an identical phenotype with CXCR5$^{-/-}$ mice, indicating that CXCL13 and CXCR5 are a monogamous chemokine and receptor pair. Using these mutants, we established that CXCL13 and CXCR5 are required for B cell homing to follicles in lymph nodes as well as in spleen and Peyer's patches. B cells fail to organize into polarized follicles in all secondary lymphoid organs in CXCL13$^{-/-}$ mice, instead forming a ring around the T zones that extends into the marginal zone in the spleen. Unexpectedly, these remaining B cell areas did not contain FDC, suggesting that lymphotoxin expression was disrupted in the B cells of CXCL13$^{-/-}$ mice. Concurrent experiments conducted by a fellow student in the laboratory, Vu Ngo, established that CXCL13 induces B cell expression of LTα1β2 and that naïve B cells express LTα1β2 in the spleen and lymph nodes of wildtype mice. Using CXCL13$^{-/-}$ mice, naïve B cell expression of LTα1β2 was found to be CXCL13-dependent, providing an explanation for the lack of FDC in B cell areas of CXCL13$^{-/-}$ spleen and lymph nodes. Collectively these data establish a model in which cytokine-induced follicular stromal cell differentiation and chemokine-directed B cell follicular homing form a positive feedback loop that controls follicle homeostasis. CXCL13$^{-/-}$ mice also lack most lymph nodes and Peyer's patches, leading to the hypothesis that a similar mechanism may control lymphoid organ development.

Despite the lack of primary follicles, germinal centers containing a high density of FDC can be induced in CXCL13$^{-/-}$ mice. Analysis of LTα1β2 expression on germinal center B cells revealed that the feedback loop that controls primary follicle homeostasis is overridden in germinal centers, with LTα1β2 expressed at a high level in a CXCL13-independent manner. The stimulus for increased LTα1β2 expression by germinal center B cells may be provided by CD40L or other signals derived from the T cells that enter follicles during T-dependent antibody responses and are necessary for germinal center function. Consistent with a role for CXCL13 in directing this change in T cell homing, we observed CXCR5 upregulation on *in vivo*-activated T cells with kinetics that correlate with T cell entry into follicles. These CXCR5^{+} T cells become responsive to CXCL13 in *in vitro* chemotaxis assays concurrent with a decrease in their responsiveness to the T zone chemokines CCL19 and CCL21. Thus reprogramming of lymphoid chemokine responses may redirect T cell homing from T zones to follicles during T-dependent immune responses and germinal center formation.

Finally, we investigated the homing properties of B1 cells, revealing an unexpected role for CXCL13 in B cell homing to body cavities. B1 cell numbers are drastically reduced in the peritoneal and pleural cavities, but not the spleen of CXCL13$^{-/-}$ mice. CXCL13 is produced by peritoneal macrophages and by radiation resistant cells in the omentum. In adoptive transfers, B1 cells home to the peritoneum in a CXCL13-dependent manner. Transferred B1 cells appear first in the omentum, indicating that this mesothelial organ serves as a port of B1

cell entry to the peritoneal cavity. CXCL13$^{-/-}$ mice have decreased natural antibody levels and are also deficient in their response against an intraperitoneally injected streptococcal vaccine. Therefore the immune function of B1 cells, like that of follicular B cells that survey secondary lymphoid organs, depends upon their CXCL13-dependent homing properties.

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Figure 1

Secondary lymphoid organ architecture.

Schematic diagrams *(A, C, E)* and immunohistochemical analysis *(B, D, F)* of sections of wildtype mouse spleen *(A-B)*, lymph node *(C-D)* and Peyer's patches *(E-F)*. Sections were stained to detect follicles (anti-IgD; brown) and T zones (anti-CD8 and anti-CD4 *(B)* or anti-CD8 alone *(D, F)*; blue). B, B cell; T, T cell; ca, central arteriole HEV, high endothelial venule. The distinct stromal and accessory cells of the follicle and T zone are represented as black shapes in schematic diagrams but are not stained in the sections.

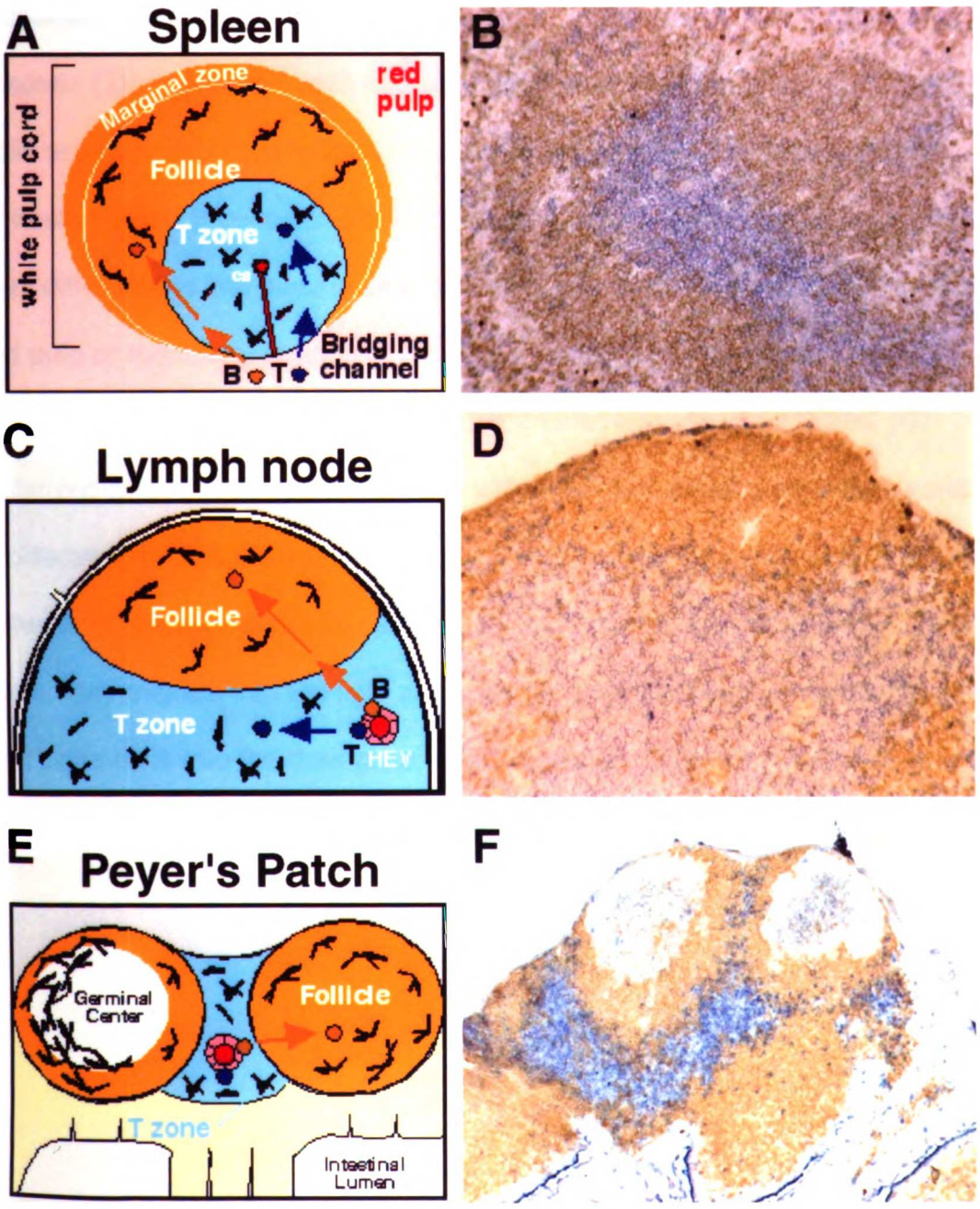
A
Spleen
white pulp cord
Marginal zone
red pulp
Follicle
T zone
CA
Bridging channel
B
T
B
C
Lymph node
Follicle
B
T zone
T
HEV
D
E
Peyer's Patch
Germinal Center
Follicle
T zone
Intestinal Lumen
F

Figure 2

Changes in lymphocyte homing during T-dependent antibody responses. Schematic representation of lymphocyte homing in draining lymph nodes after immunization. *(A)* After interaction with antigen-presenting dendritic cells (DC) in T zones (T), activated T cells migrate toward B cell follicles. Antigen stimulated B cells are excluded from follicles (F), accumulating at the T/B boundary. *(B)* Activated T and B cells interact in the outer T zones. B cells receiving T cell help may differentiate into plasma cells that migrate out of the T zone into the splenic red pulp or medullary regions of the lymph node. Germinal center B cell precursors, together with some activated T cells, migrate into follicles. *(C)* Polarized germinal centers (GC) form within follicles, developing a dark zone of proliferating B cells closest to the T zone, and a distal light zone with a dense network of follicular dendritic cells (FDC). As B cells in the dark zone exit the cell cycle, they migrate into the light zone. Some B cells may also migrate from the light zone back into the dark zone. Antigen-specific T cells are found in both the light zone and the follicular mantle (FM) at this time.

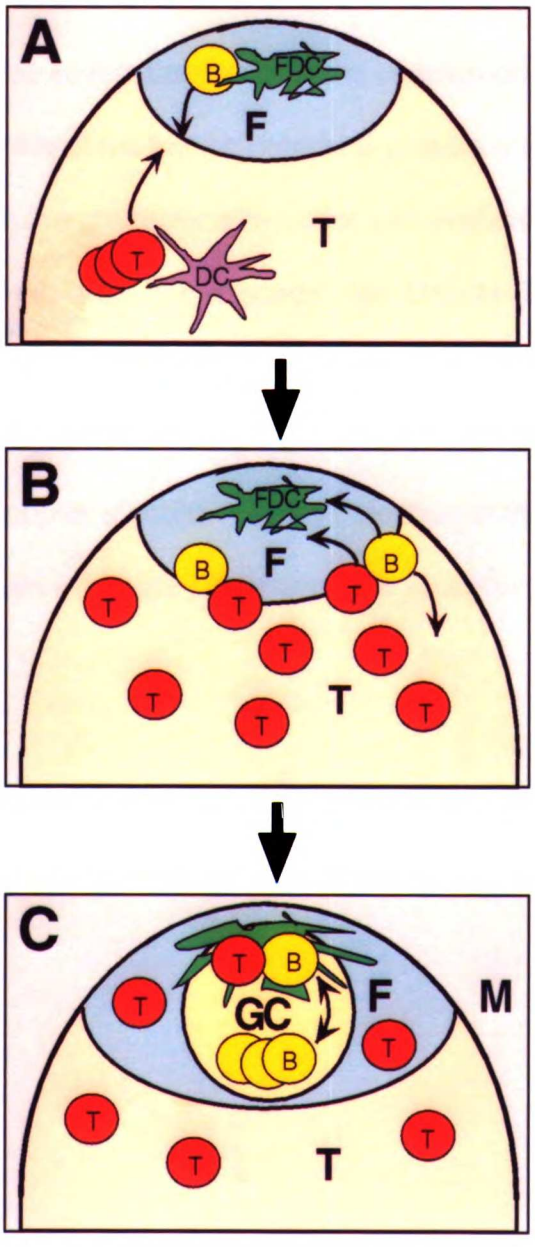
A
B
FDC
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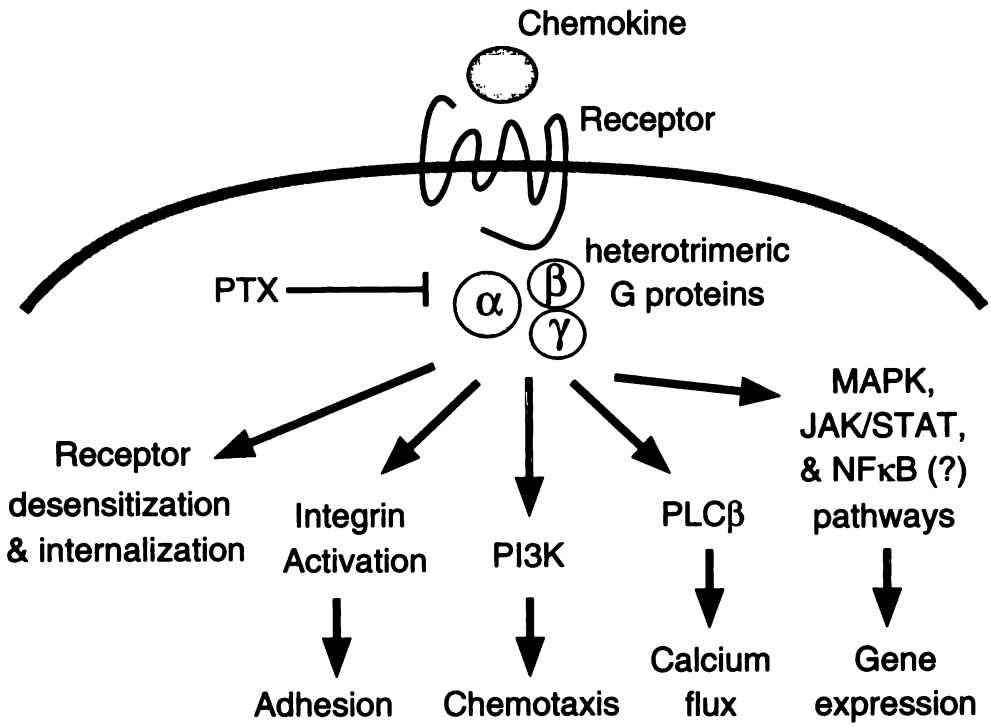
Figure 3

Schematic view of the physiological effects of chemokine receptor signaling.

Binding of chemokines to seven-transmembrane chemokine receptors activates a pertussis toxin (PTX)-sensitive heterotrimeric G protein-coupled signaling cascade. Depending on the chemokine/receptor pair and cell type, the physiological effects of this signaling cascade may include chemotaxis, rapid activation of integrin-mediated adhesion, receptor desensitization and internalization, and changes in gene expression. Calcium flux is often measured to detect chemokine receptor signals. A small selection of the signaling molecules that have been implicated in chemokine receptor signaling are shown.


Chemokine
Receptor
heterotrimeric
G proteins
PTX
α
β
γ
Receptor
desensitization
& internalization
Integrin
Activation
PI3K
PLCβ
MAPK,
JAK/STAT,
& NFκB (?)
pathways
Adhesion
Chemotaxis
Calcium
flux
Gene
expression

Chapter 2

A chemokine driven positive feedback loop organizes lymphoid follicles

Ansel, K.M., V.N. Ngo, P.L. Hyman, S.A. Luther, R. Förster, J.D. Sedgwick, J.L. Browning, M. Lipp, and J.G. Cyster. 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 406:309-14.

Summary

Lymphoid follicles are B cell-rich compartments of lymphoid organs that function as sites of B cell antigen-encounter and differentiation. CXC chemokine receptor-5 (CXCR5) is required for B cell migration to splenic follicles (1), but requirements for homing to B cell areas in lymph nodes (LNs) have not been defined. Here we show that LNs contain two types of B-cell rich compartment, follicular dendritic cell (FDC) containing follicles, and areas lacking such cells. Using gene-targeted mice, we establish that B-lymphocyte chemoattractant (BLC/BCA1 (2,3)) and its receptor, CXCR5, are needed for B cell homing to follicles in LNs as well as in spleen. We also find that BLC is required for development of most LNs and Peyer's patches. In addition to mediating chemoattraction, BLC induces B cells to upregulate membrane lymphotoxin (LT)α1β2, a cytokine that promotes FDC development and BLC expression (4,5), establishing a positive feedback loop that is likely to play a key role in follicle development and homeostasis. In germinal centers, the feedback loop is overridden with B cell LTα1β2 expression being induced by a BLC independent mechanism.

Introduction, Results, and Discussion

The BLC gene was inactivated by deletion of a portion of exon 2 which encodes amino acids 27 to 60, including three of the four conserved cysteine residues (Fig. 1a). Mice homozygous for the targeted locus (Fig. 1b) lacked detectable BLC mRNA (Fig. 1c) and protein (Fig. 1d). Anatomical analysis revealed that BLC-deficient mice had severe but incompletely penetrant defects in development of peripheral lymphoid organs. The majority of mice lacked inguinal, iliac, sacral, brachial, and axillary LNs, among others (Table 1). All of these LNs were found at varying low frequencies, however, and several other LNs developed normally, with all animals possessing a full set of mesenteric LNs (Table 1). The lymphoid patch of the cecum was also absent and the number of Peyer's patches was severely reduced (Table 1). Those Peyer's patches that were found were typically smaller and lacked the characteristic multi-domed structure of wildtype Peyer's patches. Previous characterization of CXCR5-deficient mice established that they typically lack inguinal LNs and have a less severe deficiency in Peyer's patches than BLC-deficient mice (1). Further analysis of animals on a 129 or 129xB6 mixed background indicated that they often lack additional peripheral LNs (Table 1). These findings establish a broad requirement for the BLC/CXCR5 ligand/receptor pair in one of the earliest steps in development of Peyer's patches and most LNs.

To test whether BLC is needed for organization of B cells in lymphoid follicles, sections of LNs and spleen from $BLC^{-/-}$ animals were analyzed for B cell distribution. In all these organs, B cells failed to organize in polarized follicular

clusters, and instead appeared as a ring of cells at the perimeter of T cell areas (Fig. 2a). The boundary between B cell-rich areas and T zones was often poorly demarcated, with increased numbers of B cells and T cells in reciprocal areas (Fig. 2a, b). In addition, staining spleen sections for IgM and IgD revealed a thickened ring of IgM^{hi} IgD^{lo} marginal zone B cells (Fig. 2a, upper panels). The segregation of B cells between this area and the inner ring of B cells was also disrupted, with increased numbers of IgM^{lo} IgD^{hi} B cells located in the outer marginal zone (Fig. 2a, upper panels).

A key property of B cell follicles is the presence of immune-complex-presenting FDCs (6). Staining for complement receptor-1 (CR1), which is highly expressed on FDCs and marginal zone B cells, revealed an absence of primary follicle FDCs in BLC-deficient spleen and LNs (Fig. 2b). Previous studies in CXCR5-deficient mice established a requirement for this receptor in B cell follicle formation in spleen and Peyer's patches, but did not indicate a role for the receptor in LNs (1). However, during our analysis of BLC expression in LNs, we observed two types of B cell-rich zones: polarized follicles that are BLC positive and contain FDCs but few T cells; and areas that lack staining for BLC or FDC markers, contain substantial numbers of T cells, and lack a polarized morphology (Fig. 2c,see also e, f). BLC staining was detected on a subset of $CR1^{+}$ FDCs as well as on adjacent $CR1^{-}$ follicular stromal cells (Fig. 2d). Analysis of LNs from $CXCR5^{-/-}$ animals for these two types of B cell areas revealed that they lack FDC-containing primary follicles (data not shown). In transfer experiments, $CXCR5^{+/+}$ B cells localized in FDC-containing follicles and FDC-deficient B cell areas of

wildtype LNs (Fig. 2e). By contrast, $CXCR5^{-/-}$ B cells failed to enter FDC-containing follicles (Fig. 2f). $CXCR5^{-/-}$ B cells also did not migrate into follicles in the spleen and Peyer's patches, as previously observed (1); however, $CXCR5^{-/-}$ B cells did localize in the FDC-deficient B cell-rich areas in LNs (Fig. 2f). B cell homing to LN follicles therefore requires BLC and CXCR5, but neither molecule is necessary for migration to FDC-deficient B-cell rich regions of secondary lymphoid tissues.

A lack of FDCs and organized follicles, as well as impaired development of multiple lymphoid organs, are phenotypes shared between BLC- and LT-deficient mice (4). Cell transfer experiments showed that B cells are an essential source of $LT\alpha1\beta2$ for development of FDCs, but it was not clear whether $LT\alpha1\beta2$ was expressed by naïve B cells (7-9). Although B cells express $LT\beta$ constitutively (10-12), surface expression of this subunit requires $LT\alpha$, and $LT\alpha$ expression has only been reported following exposure to activating stimuli such as CD40L or IL-4 (11) or treatment with endotoxin or phorbol esters (13). However, development of primary follicles is not associated with B cell activation, as it occurs in germ-free mice (14), immunoglobulin (Ig)-transgenic mice (15), T cell deficient mice (16), CD40-deficient mice (17) and IL4-deficient mice (18). To investigate $LT\alpha1\beta2$ expression on naïve B cells we used a soluble form of the $LT\beta$ receptor, $LT\beta R$-Ig, to stain freshly isolated cells (Fig. 3a-g). Strikingly, a significant fraction of total B cells from wildtype spleen showed low but detectable $LT\beta R$-Ig binding compared to $LT\alpha$- or $LT\beta$-deficient spleen cells (Fig. 3c, d, g). An even larger fraction of LN B cells stained with $LT\beta R$-Ig (Fig. 3e, f, g) whereas B cells in the blood (Fig. 3a,

b, g) and bone marrow (not shown) were mostly negative. Similar levels of LTα1β2 were observed on B cells in Ig-transgenic animals (Fig. 3g), demonstrating that the expression was not induced by antigen. We then tested LTα1β2 expression on B cells in BLC-deficient animals (Fig. 3h). While LTα1β2 expression on $BLC^{-/-}$ peripheral blood B cells was not different from wildtype controls, expression on B cells from spleen and LNs was significantly reduced (Fig. 3h), establishing that BLC is required for normal LTα1β2 expression on naïve B cells.

To determine whether recirculating B cells upregulated LTα1β2 expression as they moved into BLC-expressing lymphoid organs, cells were isolated from the blood of $Ly5.1^{+}$ donor mice and transferred into congenic $Ly5.2^{+}$ recipients. At 0.5 and 6 hrs after transfer, recipient splenocytes were harvested and stained with anti-Ly5.1 to detect the transferred cells, and with LTβR-Ig (Fig. 3i). At the early time point, when most of the transferred B cells in the spleen are in the non-lymphoid area (not shown), few of the cells expressed LTα1β2 (Fig. 3i). However, six hrs post-transfer, when many of the transferred cells are in BLC^{+} follicular areas (not shown), LTα1β2 expression was readily detectable (Fig. 3i). When B cells were taken from spleen and transferred intravenously to syngeneic recipients, LTβR-Ig staining of cells in recipient blood and spleen soon after transfer had diminished to levels similar to endogenous blood B cells (Fig 3i). The mechanism for this downregulation is not yet understood, but it suggests that as B cells leave a lymphoid organ and enter the blood they rapidly lose surface LTα1β2 expression. In practical terms, this finding allowed us to use cells

from spleen for further transfer experiments to test the mechanism of LTα1β2 upregulation on cells entering lymphoid organs. Within six hrs of transfer, untreated spleen B cells expressed amounts of LTα1β2 equal to endogenous B cells (Fig. 3i). By contrast, cells pretreated with pertussis toxin (PTX), an inhibitor of signaling by Gαi coupled receptors, including all known chemokine receptors, failed to upregulate LTα1β2 expression after transfer (Fig. 3i). Furthermore, minimal LTα1β2 upregulation occurred when B cells from wildtype mice were transferred to BLC-deficient recipients (Fig. 3j, k). Interestingly, when B cells from BLC-deficient donors were transferred to wildtype recipients, an exaggerated induction of LTα1β2 occurred (Fig. 3l, m), perhaps because B cells that develop in BLC-deficient animals are hypersensitive to the chemokine. Taken together, these findings provide strong support for the hypothesis that recirculating B cells upregulate LTα1β2 as they migrate to B cell areas in response to BLC.

To test whether BLC functions directly to induce LTα1β2 expression, we incubated naïve B cells *in vitro* with recombinant BLC (Fig. 4). Strikingly, BLC induced a dose sensitive upregulation of LTα1β2 on cultured B cells (Fig. 4a). The induction was inhibited by preincubation of the cells with PTX (Fig. 4b). Three other chemokines with chemotactic activity on naïve B cells were also tested: the broadly expressed CXCR4 ligand, SDF1; and SLC and ELC, two related CC chemokines made in the T cell area that are ligands for CCR7 (19). Each of these chemokines caused detectable increases in LTα1β2 expression on B cells, though in all cases the maximal induction was lower than the induction by BLC (Fig. 4c and data not shown). While it is not yet clear if these chemokines

can induce LTα1β2 expression on naïve B cells in vivo, it seems possible that they contribute to the remaining expression in $BLC^{-/-}$ mice (Fig. 3h). BLC mediated induction of LTα1β2 was sensitive to actinomycin D, cycloheximide and wortmannin (Fig. 4d) providing evidence that BLC may signal via phosphatidylinositon-3-OH kinase to induce lymphotoxin transcription.

Despite low expression of LTα1β2 on naïve B cells and the absence of primary follicle FDCs in BLC-deficient mice, germinal centers formed in LNs and spleen following immunization with a T-dependent antigen (Fig. 5a). These germinal centers were misplaced and usually smaller than those found in wildtype controls, yet they contained $CR1^{+}$ FDC networks (Fig. 5a). Similar observations for spleen germinal center formation were recently made in CXCR5-deficient mice (20). Germinal center FDCs, like those of primary follicles, require LTα1β2 for their development and maintenance (4, 21). Staining with LTβR-Ig revealed high LTα1β2 expression on $GL7^{+}$ germinal center B cells in wildtype mice (Fig. 5b). In striking contrast to our observations for naïve B cells, LTα1β2 expression on germinal center B cells was not BLC dependent (Fig. 5b). We conclude that the requirement for BLC to induce LTα1β2 is overridden in germinal centers, most likely by T cell derived signals. CD40 signaling is necessary for germinal center formation, and might provide the required signal for inducing LTα1β2. In agreement with this possibility, CD40-mediated induction of LTα1β2 is CXCR5-independent (Fig. 5c).

In summary, our findings indicate that BLC has dual roles in follicular compartmentalization of B cells: mediating B cell attraction, and inducing

increased LTα1β2 expression on the recruited cells. LTα1β2 then engages LTβRs on non-hematopoietic stromal cells, promoting maturation of FDCs and leading to increased expression of BLC (4, 5). FDC maturation is regulated by signals in addition to LTα1β2, including signals from TNF/TNFR1 (4), and it remains to be determined whether these molecules function in the same or parallel pathways. In addition to demonstrating expression of LTα1β2 on naïve B cells, this work establishes the existence of a positive feedback loop between BLC and LTα1β2. This feedback loop is likely to be critical in follicle development, causing the low amounts of BLC that are expressed independently of LTα1β2 (5) to become upregulated as B cells are recruited. When the number of B cells entering an adult lymphoid organ increases, for example during an immune response, the feedback loop may help ensure that the follicular compartment can expand and accommodate the increased numbers of B cells. BLC-mediated B cell recruitment and LTα1β2 induction may also play a role in Peyer's patch organogenesis since, in addition to requiring BLC and LTα1β2, these structures depend strongly on B cells (22). In support of this, ectopic expression of BLC induces B cell- and LTα1β2-dependent lymphoid neogenesis (23). A very early step in development of Peyer's patches and LNs is local accumulation of $CD3^-IL7R^+$ cells that express LTα1β2 (24-26). As some of these cells express CXCR5 (27), we propose that BLC functions in LN development by recruiting $CXCR5^+CD3^-IL7R^+$ cells and inducing them to upregulate LTα1β2. Finally, the finding that germinal center B cells express LTα1β2 and that this expression is BLC-independent, together with the detection of $CR1^+$ FDCs in

germinal centers of $BLC^{-/-}$ mice, reveals that the pathway controlling FDC development in germinal centers is distinct from the BLC-mediated feedback loop operating in primary follicles.

Methods

Generation of $BLC^{-/-}$ mice.

A 10 kb EcoRI fragment containing the *blc* gene was isolated from a 129 mouse genomic DNA library (Genome Systems). A targeting vector was constructed in which nucleotides 18 to 116 of the second exon of *blc* were replaced with an in-frame stop codon followed by a Mengo virus internal ribosomal entry site, the gene encoding enhanced green fluorescent protein and, in reverse orientation, a loxP-flanked neomycin resistance gene (*neo*^{r}). The linearized construct was electroporated into JM1 129 mouse ES cells (a kind gift of N. Killeen). G418 (200μg/mL) resistant colonies were screened by Southern hybridization of EcoRI-digested genomic DNA to a flanking 1.0kb probe. Targeted clones were injected into C57BL/6 (B6) blastocysts. Chimeric males were mated to B6 females, and germline transmission of the targeted allele was detected by Southern blot and by PCR using the primers 5'-CGTCTATGTTCTTTGTCCAATGGG-3' (sense), 5'-CTTACACAACTTCAGTTTTGGGGC-3' (antisense; wild-type), and 5'-ACCTTGTATTCCTTTGTCGAGAGG-3' (antisense; mutant). Homozygous mutant mice were born at Mendelian ratios, were fertile, and appeared healthy. Most mice used in this study were mixed 129 and B6 strain background carrying

the original targeted allele, although the phenotype of these mice was indistinguishable from mice homozygous for a *neo*^{r}-deleted allele. Northern blot analysis was as described (5) using probes specific for *blc* exon 2 or *EF-1α*. BLC protein was detected in heparin-sepharose precipitates of 1% NP40 spleen homogenates by Western blotting with polyclonal goat anti-mouse BLC (R&D Systems). B6 wildtype mice and some of the LTα$^{-/-}$ mice (on a mixed 129/B6 background (5, 28)) were from Jackson Labs (Bar Harbor, Maine). Other LTα$^{-/-}$ mice and the LTβ$^{-/-}$ mice were from a B6 colony (29). Ig-transgenic mice were of the MD4 line (15, 29). To induce germinal centers, mice were injected intraperitoneally with 100μg of TNP- or NP-chicken gamma globulin (Biosearch Technologies) diluted in 200μL PBS and mixed with the Ribi Adjuvant System (Ribi Immunochem Research).

Flow cytometry, tissue staining and adoptive transfers.

Surface expression of LTα1β2 was detected with LTβR-Ig (13), followed by anti-human IgG-PE (Jackson ImmunoResearch). Free IgG binding sites were blocked by incubation with mouse and rat serum (1:25 dilution) for 15 min. Cells were then stained with appropriate antibody combinations for identifying lymphocyte subsets. Anti-LTβ pre-blocking by incubation with 10 μg/ml BBF6 (13) for 20 min inhibited LTβR-Ig staining of naïve and germinal center B cells. Flow cytometry, *in situ* hybridization, immunohistochemistry, and immunofluorescence microscopy were as described (19). For simultaneous detection of BLC and FDC, sections were incubated with goat anti-mBLC

followed by donkey anti-goat IgG-biotin (Jackson). After color development, sections were additionally stained with anti-CD35 (8C12). Anti-sheep IgG, anti-Armenian hamster IgG, anti-rat IgG-Cy3 and -HRP (Jackson), anti-rat IgG-biotin (Vector), anti-mouse IgD (Binding Site), anti-mouse IgM, anti-B220, anti-CD4, anti-CD8 (Caltag), and all other antibodies (Pharmingen) were purchased, except anti-CD3 (2C11), a kind gift of L. Zuckerman. Biotinylated reagents were detected with streptavidin-Cychrome (flow cytometry; Pharmingen), Vectastain ABC-AP (immunohistochemistry; Vector) or streptavidin-AMCA (immunofluorescence; Vector). Adoptive transfers were as described(19). In some experiments, donor cells were preincubated at 10^7/ml at 37°C for 1 hr with 200ng/ml PTX (List Biological Laboratories). Splenic B cells from CXCR5$^{-/-}$ or wildtype donors were purified by staining non-B cells with anti-CD43-biotin (Pharmingen), followed by MACS® depletion and CFSE labeling as described (19).

Chemokines and cell culture.

Recombinant mouse chemokines were produced and used in chemotaxis assays as described (19). In preliminary experiments, *in vitro* incubation of B cells was found to cause rapid upregulation of LTα1β2, but after further culture expression returned to levels similar to freshly isolated cells (data not shown). Therefore, B6 spleen cells (10^6 /well) were pre-incubated in flat bottom 96-well plates at 37°C, 5% CO_2 in 200 µl of RPMI / 10% fetal calf serum for 14-15 hrs to allow adjustment to *in vitro* conditions. Chemokines or anti-CD40 (FGK; a kind

gift of A. Rolink) was then added in 20µl of medium. In some experiments PTX, actinomycin D (Calbiochem), cycloheximide (Calbiochem) or wortmannin (Sigma) was also added. Six hours later, cells were washed and analyzed by flow cytometry. None of the chemokine preparations were found to cause upregulation of the activation marker CD69.

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Table 1

Defective lymph node and Peyer's patch development in BLC and CXCR5-deficient mice.

$BLC^{+/+}$, $BLC^{+/-}$, $BLC^{-/-}$ and $CXCR5^{-/-}$ mice on a mixed B6/129 strain background were examined for the presence of LNs and PPs.

* For LNs, data shown are of the form *x/n*, where *x* is the number of mice in which the respective lymph nodes were identified out of *n* mice examined. For nodes that develop in two symmetrical sites, the number in parentheses represents the number of mice in which a LN had developed in one of the two sites. The complete absence of lymph nodes was confirmed in some mice by intraperitoneal injection of 300μL 1% Pontamine Blue dye in sterile PBS 10-20 days before dissection. The dye becomes concentrated in lymphoid organs as it is cleared from surrounding tissues, allowing easy detection of even minute lymphoid aggregates (30).

† For PPs, data shown are the number of mice in which the indicated number of PPs was identified along the small intestine.

‡ Ten additional $CXCR5^{-/-}$ mice on the 129 strain background (six times backcrossed) were examined, revealing strain-dependent differences in LN development.

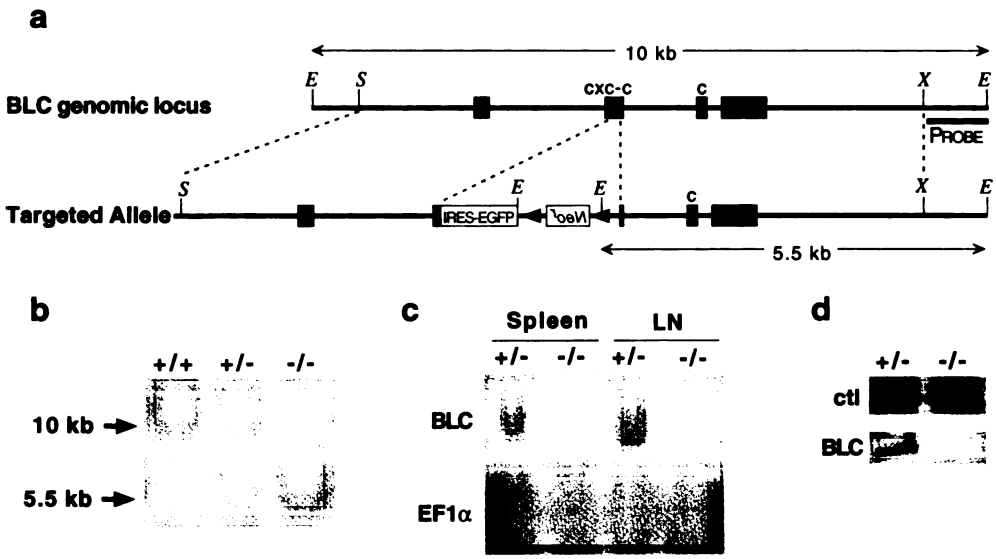
Defective lymph node and Peyer's patch development in BLC and CXCR5-deficient mice

Genotype	Lymph Nodes*												Peyer's Patches†			
	inguinal	iliac/ periaortic	axillary	brachial	popliteal	deep cervical	superficial cervical	facial	renal	mesenteric	sacral/ caudal	parathymic	0	1-2	3-6	>6
$BLC^{+/\pm}$	41(0)/41	38(0)/38	42(0)/42	42(0)/42	15(2)/19	14(2)/16	47(0)/47	47(0)/47	12(3)/15	50/50	37/38	15/15	0	0	2	29
$BLC^{-/-}$	0(2)/41	0(0)/41	1(5)/42	1(8)/42	0(0)/18	1(2)/14	42(1)/46	41(2)/46	0(0)/12	50/50	1/40	0/14	21	11	5	0
$CXCR5^{-/-}$	0(0)/5	0(0)/5	0(2)/5	2(0)/5	0(0)/5	0(0)/5	5(0)/5	3(1)/5	0(0)/5	5/5	0/5	0/5	1	2	1	1
$CXCR5^{-/-}$‡	0(0)/10	0(0)/10	0(1)/10	10(0)/10	0(0)/10	0(1)/10	9(1)/10	10(0)/10	3(3)/9	10/10	1/10	0/10	0	5	3	1

Figure 1

BLC genomic locus and lack of BLC mRNA and protein in BLC$^{-/-}$ mice.

(a), BLC genomic locus before and after gene targeting. Black boxes indicate exons (1-4). The four cysteine residues are encoded by exons 2 and 3 as indicated. E, EcoR1; S, SacI; X, XhoI. *(b)*, Southern blot analysis of EcoR1-digested genomic DNA from +/+, +/- and -/- mice. *(c)*, Northern blot of total RNA from +/- and -/- spleen and mesenteric LN. *(d)*, Western blot of heparin-precipitates from +/- and -/- spleen lysate for BLC. ctl, non-specific band detected with anti-BLC serum, shown as a loading control.


a
10 kb
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E
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IRES-EGFP
5.5 kb
b
+/+
+/-
-/-
10 kb
5.5 kb
c
Spleen
LN
+/-
-/-
+/-
-/-
BLC
EF1α
d
+/-
-/-
ctl
BLC

Figure 2

Absence of primary follicles and FDCs in BLC$^{-/-}$ mice and defective homing of CXCR5$^{-/-}$ B cells to LN follicles.

(a), BLC$^{+/-}$ and $^{-/-}$ spleen and LN stained for the indicated markers. The color of the labels corresponds to the color of the reaction product for that marker. Follicular B cells are mostly IgD^{hi} IgM^{lo} and appear brown, whereas marginal zone B cells are mostly IgD^{lo} IgM^{hi} and appear blue (upper panels).The boundaries of the marginal zone are indicated by brackets. *(b)*, BLC$^{+/-}$ and $^{-/-}$ spleen and LN stained to detect CR1 and T cells. FDCs appear as a network of intense staining for CR1, whereas splenic CR1^{hi} marginal zone B cells appear as a rim of faintly stained cells. Spleen and LNs of more than twenty BLC$^{+/-}$ and $^{-/-}$ mice were analyzed for follicular organization. *(c)*, Wildtype (WT) LN stained to detect BLC and B cells. Inset: Adjacent section stained to detect T cells. *(d)*, High magnification view of a wildtype LN follicle stained to detect BLC and FDC (CR1). Arrows indicate examples of BLC and CR1 double positive cells. Data are representative of 4 independent experiments. *(e,f)*, Distribution of transferred wildtype *(e)* or CXCR5$^{-/-}$ *(f)* CFSE-labeled B cells in LNs of wildtype recipients. Sections are stained to detect endogenous B cells and FDCs. Data are representative of four recipients. F, follicle; T, T zone; B*, BLC- and FDC-deficient area rich in B cells.

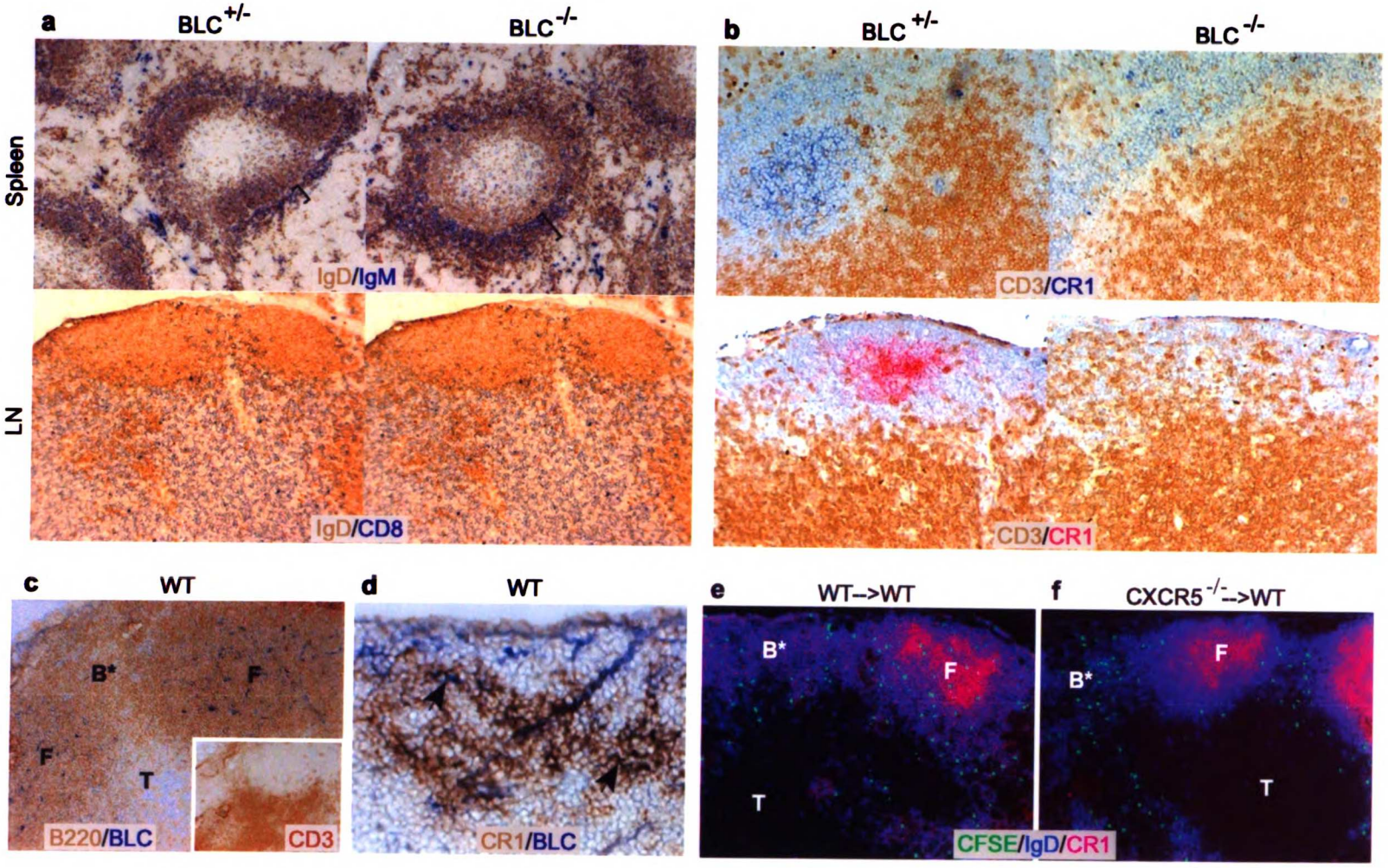

a
BLC+/-
BLC-/-
Spleen
IgD/IgM
LN
IgD/CD8
b
BLC +/-
BLC -/-
CD3/CR1
CD3/CR1
c
WT
B*
F
F
T
B220/BLC
CD3
d
WT
CR1/BLC
e
WT–>WT
B*
F
T
f
CXCR5-/-–>WT
B*
F
T
CFSE/IgD/CR1

Figure 3

Membrane LTα1β2 expression on B cells in peripheral lymphoid tissues and dependence on BLC.

(a-f), Flow cytometric analysis of cells from wildtype (left panels) and $LT\alpha^{-/-}$ (right panels) blood *(a, b)*, spleen *(c, d)*, and LNs *(e, f)* stained for LTβR-Ig and B220. In *(f)*, LN cells were from irradiated wildtype mice that had been reconstituted for 8 weeks with $LT\alpha^{-/-}$ bone marrow. Gates with solid lines show LTβR-Ig^{hi} B cells; total B cells were calculated as the cells in the region bounded by the dashed lines plus the cells within the LTβR-Ig^{hi} gate. Percentages of LTβR-Ig^{hi} B cells as a fraction of total B cells are shown above the windows. *(g)*, Summarized data from multiple flow cytometric analyses as shown in *(a-f)* with additional data obtained from $LT\alpha^{-/-}$ and HEL-specific Ig-transgenic mice. *(h)*, LTα1β2 expression levels on B cells from blood, spleen and mesenteric LNs (mLN) of $BLC^{-/-}$ animals compared with B cells of $BLC^{+/-}$ littermate controls. Cells were gated as in *(a-f)*. Bars represent the mean ± standard deviation of percentages of LTβR-Ig^{hi} B cells for the indicated number of animals. Statistical comparison is by Student's t-test. **i**, Upregulation of LTα1β2 on B cells migrating into spleen occurs in a PTX sensitive manner. Each point represents an individual recipient and bars represent the mean percentages of LTβR-Ig^{hi} B cells. *(j-m)*, LTα1β2 upregulation on transferred B cells is BLC dependent. Splenocyte donors and transfer recipients were as follows: *(j)*, $Ly5.1^{+}$ wildtype into $BLC^{+/-}$. *(k)*, $Ly5.1^{+}$ wildtype into $BLC^{-/-}$. *(l)*, $Ly9.1^{+}$ $LT\alpha^{-/-}$ into $Ly9.2^{+}$ wildtype. *(m)*, $Ly9.1^{+}$ $BLC^{-/-}$ into $Ly9.2^{+}$ wildtype. Data shown are LTβR-Ig and B220 staining of transferred $Ly5.1^{+}$

(j, k) or Ly9.1^{+} *(l, m)* cells in recipient spleens 6 hrs after transfer, and are representative of three transfers of each type.

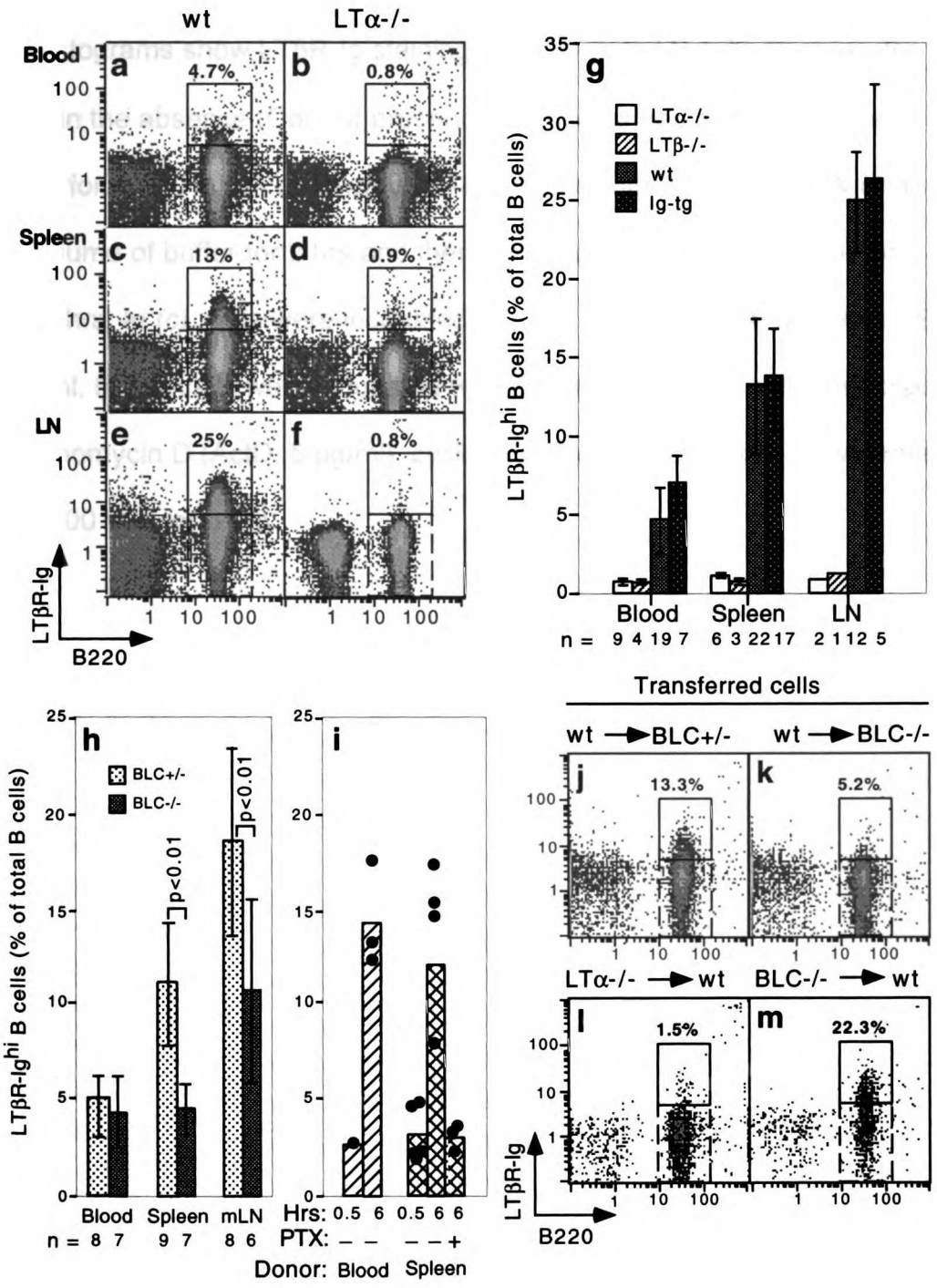

wt
LTα-/-
Blood
Spleen
LN
a
b
c
d
e
f
4.7%
0.8%
13%
0.9%
25%
0.8%
LTβR-Ig
B220
g
LTα-/-
LTβ-/-
wt
Ig-tg
LTβR-Ig^hi B cells (% of total B cells)
Blood Spleen LN
n = 9 4 19 7 6 3 22 17 2 1 12 5
h
BLC+/-
BLC-/-
p<0.01
p<0.01
LTβR-Ig^hi B cells (% of total B cells)
Blood Spleen mLN
n = 8 7 9 7 8 6
i
Hrs: 0.5 6 0.5 6 6
PTX: – – – – +
Donor: Blood Spleen
Transferred cells
wt → BLC+/-
wt → BLC-/-
j
k
13.3%
5.2%
LTα-/- → wt
BLC-/- → wt
l
m
1.5%
22.3%
LTβR-Ig
B220

Figure 4

BLC induces LTα1β2 up-regulation on B cells *in vitro*.

(a-d), Histograms show LTβR-Ig staining of B220-gated B cells from *in vitro* cultures in the absence (Con) or presence of chemokines at the indicated amounts for 6 hrs. In *(b)*, cells were pre-incubated with 200 ng/ml PTX or an equal volume of buffer for 2 hrs and then incubated with BLC (3 μg/ml) for a further 6 hrs. In *(c)*, cells were incubated in SLC, ELC or BLC at 3 μg/ml or SDF1 at 1 μg/ml. In *(d)*, cells were incubated with BLC (3 μg/ml) alone or combined with actinomycin D (ActD, 5 μg/ml), cycloheximide (CHX, 1 μg/ml) or wortmannin (WMN, 100 nM) for 6 hrs.

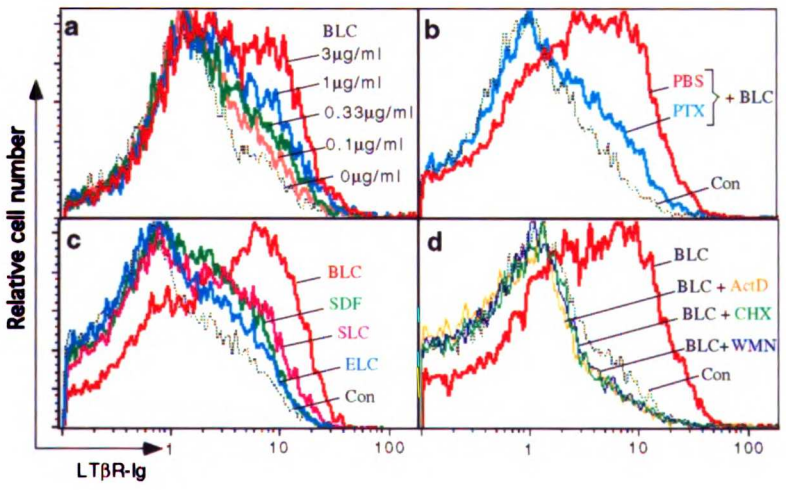
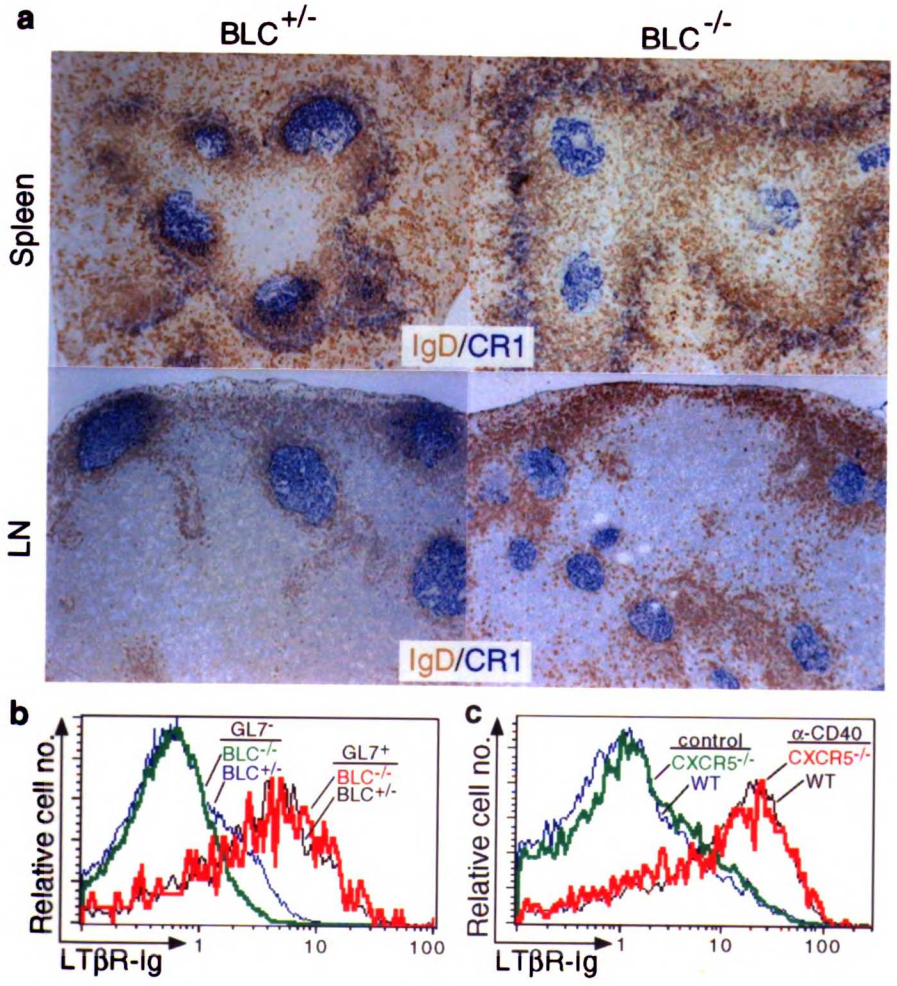

a
BLC
3μg/ml
1μg/ml
0.33μg/ml
0.1μg/ml
0μg/ml
b
PBS
PTX
+ BLC
Con
c
BLC
SDF
SLC
ELC
Con
d
BLC
BLC + ActD
BLC + CHX
BLC+ WMN
Con
Relative cell number
1
10
100
LTβR-Ig

Figure 5

Misplaced FDC-containing germinal centers in BLC$^{-/-}$ mice and BLC-independent expression of LTα1β2 on germinal center B cells.

(a), Spleen and LN from immunized mice stained to detect non-germinal center B cells (IgD) and FDCs (CR1). Staining of serial sections with peanut agglutinin (PNA), established that the strongly-CR1^{+} areas in the BLC$^{-/-}$ spleen and LN were germinal centers (not shown). Data are representative of 8 immunized BLC$^{+/-}$ and $^{-/-}$ mice. *(b)*, LTα1β2 expression on germinal center B cells. Histograms show LTβR-Ig staining on gated B220^{+}GL7^{-} non-germinal center and B220^{+}GL7^{+} germinal center B cells from mesenteric LNs of immunized BLC$^{+/-}$ and $^{-/-}$ mice. *(c)*, *In vitro* CD40 stimulation induces LTα1β2 expression in a CXCR5-independent manner. Spleen cells were incubated with buffer alone (control) or anti-CD40 antibody for 6 hrs and then stained. Histograms show LTβR-Ig staining on B220^{+} cells.


a
BLC+/-
BLC-/-
Spleen
IgD/CR1
LN
IgD/CR1
b
Relative cell no.
GL7-
BLC-/-
BLC+/-
GL7+
BLC-/-
BLC+/-
LTβR-Ig
1
10
100
c
Relative cell no.
control
CXCR5-/-
WT
α-CD40
CXCR5-/-
WT
LTβR-Ig
1
10
100

Chapter 3

In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines

Ansel K.M., L.J. McHeyzer-Williams, V.N. Ngo, M.G. McHeyzer-Williams, and J.G. Cyster. 1999. *In vivo*-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J. Exp. Med.* 190:1123-34.

Summary

Migration of antigen-activated CD4 T cells to B cell areas of lymphoid tissues is important for mounting T-dependent antibody responses. Here we show that CXC chemokine receptor (CXCR)-5, the receptor for B-lymphocyte chemoattractant (BLC), is upregulated on antigen-specific CD4 T cells *in vivo* when animals are immunized under conditions that promote T cell migration to follicles. *In situ* hybridization of secondary follicles for BLC showed high expression in mantle zones and low expression in germinal centers (GCs). When tested directly *ex vivo*, $CXCR5^{hi}$ T cells exhibited a vigorous chemotactic response to BLC. At the same time, the $CXCR5^{hi}$ cells showed reduced responsiveness to the T zone chemokines, EBV-induced molecule-1 (EBI-1) ligand chemokine (ELC) and secondary lymphoid tissue chemokine (SLC). Following adoptive transfer, $CXCR5^{hi}$ CD4 T cells did not migrate to follicles indicating that additional changes may occur following immunization that help direct T cells to follicles. To further explore whether T cells could acquire an intrinsic ability to migrate to follicles, $CD4^{-}CD8^{-}$ double negative (DN) T cells from MRL-lpr mice were studied. These T cells normally accumulate within follicles of MRL-lpr mice. Upon transfer to wild-type recipients, DN T cells migrated to follicle proximal regions in all secondary lymphoid tissues. Taken together, our findings indicate that reprogramming of responsiveness to constitutively expressed lymphoid tissue chemokines plays an important role in T cell migration to the B cell compartment of lymphoid tissues.

Introduction

T cell dependent antibody responses require cognate interaction between antigen-presenting B cells and antigen-specific T cells (1, 2). Antigen mediated cross-linking of the B cell receptor (BCR) promotes B cell localization at the boundary between T zone and follicles (reviewed in (3)), the location of the earliest encounters between antigen-bearing B cells and antigen specific T cells (4-6). After receiving signals from activated CD4 T cells, some B cells undergo differentiation into plasmablasts which migrate to the red pulp of spleen or medullary cords of lymph nodes (LNs) and become antibody secreting plasma cells. Other activated B cells move into the follicle and form a germinal center (1). The germinal center is strongly dependent on help from antigen-specific T cells that must migrate into the follicle to support this response (7-12). Movement of antigen specific T cells has been studied within lymphoid tissues using an adoptive T cell transfer model (13). When recipients of OVA-specific TCR-transgenic $CD4^+$ T cells are immunized with OVA in adjuvant, there is a short period of transgenic T cell proliferation in the T zone, and then large numbers of these cells are induced to migrate into follicles (13). T cell migration to follicles has also been studied in non-transgenic mice during the response to pigeon cytochrome c (PCC) (7, 9, 14). The predominant responding T cell population expresses TCR containing Vα11 and Vβ3 (15, 16). When mice are immunized with PCC in adjuvant, a protocol that induces a strong germinal center response, Vα11Vβ3-expressing T cells are found to begin localizing in follicles by day 5 and to peak in number at day 10, with many of the cells at this

time being resident in germinal centers (7, 9, 10, 14, 17). In contrast to these findings, when mice are given antigen in saline intravenously, T cells become activated but fail to migrate into follicles. Many of the cells are quickly eliminated and those that remain respond poorly to subsequent antigen exposure (13). CD4 T cell migration into follicles has therefore been suggested not only to be important for supporting B cell responses, but also for supporting a fully developed T cell response and induction of T cell memory.

The understanding of how antigen-specific T cells migrate to follicles is poorly developed. Recently, several chemokines have been characterized that are constitutively expressed in lymphoid tissues and that help direct the movements of resting and activated lymphocytes. B-lymphocyte chemoattractant (BLC (18); also called B cell-attracting chemokine (BCA)-1 (19)) is a CXC-chemokine made by stromal cells in lymphoid follicles that functions as a ligand for CXCR5 (previously called BLR1 (20)). Gene knockout studies have established that CXCR5 is required for B cell homing to follicles in spleen and Peyer's patches (21). *In vitro*, BLC was shown to be an efficacious attractant of B cells while attracting few T cells and no myeloid cells (18, 19). Two CC-chemokines, secondary lymphoid tissue chemokine (SLC, also called 6-Ckine) and EBV-induced molecule 1 ligand chemokine (ELC, also called macrophage inflammatory protein (MIP)-3α), are constitutively expressed by cells in the T zone (22, 23). SLC and ELC are ligands for CCR7 and both strongly attract resting and *in vitro* activated T cells (22-30). Mice that lack SLC expression (and

have reduced ELC expression) have defective homing of T cells into LNs and splenic T cell areas (31).

Previous studies have shown that while most T cells are negative for CXCR5, a subset of memory phenotype cells are CXCR5-positive (20, 21). Here we have investigated the relationship between acquisition of CXCR5 expression by CD4 T cells and homing to lymphoid follicles. We establish that CXCR5 upregulation and acquisition of BLC responsiveness of *in vivo* activated CD4 T cells occurs with a time course consistent with a role in directing T cells to follicles. We also establish that at the same time as upregulating CXCR5, *in vivo* activated CD4 T cells downregulate their response to ELC and SLC. Finally, we demonstrate that DN T cells from MRL-lpr mice express CXCR5 and, upon transfer to normal recipients, migrate to follicle proximal locations in all secondary lymphoid tissues, establishing that T cells can acquire the intrinsic ability to migrate to B cell follicles.

Results

Immunization conditions that promote antigen-specific CD4 T cell homing to follicles cause rapid upregulation of CXCR5 expression

Mice that had received an inoculum of OVA-specific TCR transgenic (DO11.10) T cells were injected with $OVA_{323-339}$ peptide either s.c. in Complete Freund's Adjuvant (CFA) to promote T cell trafficking to follicles, or i.v. in PBS to promote transient T cell activation without migration to follicles (13). At days 2, 3, 5 and 10 after immunization, LN were isolated and analyzed by flow cytometry

with a clonotypic antibody, KJ1-26, that recognizes the transferred OVA-specific T cells, and with an antiserum specific for CXCR5 (35, 36). Prior to immunization, transferred OVA-specific T cells were uniformly $CXCR5^{lo/-}$ (Fig. 1A). However, within two days of immunization with peptide in CFA, when OVA-specific T cell numbers in the draining LNs start to increase (13), a subpopulation of $CXCR5^{+}$ cells could be identified (Fig. 1B). By day 3, when the OVA-specific T cell frequency had increased ~30-fold, as in previous studies (13), most of the antigen-specific cells expressed high levels of CXCR5 (fluorescence intensity at least 10-fold greater than the staining control), and after 5 days the cells were uniformly $CXCR5^{hi}$ (Fig. 1C and D). Appearance of T cells in follicles followed similar kinetics to the CXCR5 upregulation (Fig. 2). Consistent with previous reports (13, 34), KJ1-26^{+} OVA specific T cells began appearing in follicles by day 3 after immunization (Fig. 2A) and reached maximal numbers by day 5 (Fig. 2B). An enlargement of draining LN B cell areas occurred over this time period, and by day 5 many of the follicles contained nascent GCs (Fig. 2B). Immunization of mice with OVA protein in lipopolysaccharide (LPS), a protocol that has been shown to promote T cell trafficking to follicles (34), also lead to increased expression of CXCR5 on CD4 T cells (Fig. 1F). In contrast with these effects, i.v. injection of OVA peptide in saline led to only weak induction of CXCR5 on OVA specific T cells in LNs (Fig. 1A-E) and spleen (data not shown) and did not promote KJ1-26^{+} T cell migration into follicles (Fig. 2C). When mice immunized with OVA peptide in CFA were followed for longer times, a decline in CXCR5 expression was found to occur, although a significant proportion of KJ1-26^{+} cells

remained CXCR5^{hi} at day 10 post immunization (Fig 1E) and CXCR5^{hi} cells could still be detected at day 25 (data not shown). By day 10 many B cell areas had become secondary follicles, comprising a well-developed germinal center and a surrounding mantle of small resting B cells (Fig 2D). Significant numbers of KJ1-26^{+} T cells were detectable in the secondary follicles, with many residing in the mantle zone and smaller numbers being associated with the outer zone of germinal centers (Fig 2D). BLC *in situ* hybridization analysis of LNs containing well developed GCs showed BLC was highly expressed in the follicular mantle zones (Fig. 2E, F). Within GCs, only occasional cells could be identified that hybridized with the BLC probe and these cells tended to be most frequent in the area of the GC distal to the T zone (Fig. 2E, F). This is also the region of the GC most enriched for CD4 T cells (Fig. 2D and (1)).

CXCR5 upregulation on Vα11Vβ3-expressing cells during the PCC response

To test whether CXCR5 was upregulated on non-transgenic T cells under conditions not involving T cell transfer, the phenotype of PCC-specific Vα11Vβ3-expressing CD4 T cells was followed in mice immunized s.c. with PCC protein in adjuvant. Vα11Vβ3-expressing T cells responding to PCC were detected in immunized, but not unimmunized animals by upregulation of CD44 (Fig. 3A). CXCR5 expression became detectable on a subset of PCC-responsive Vα11^{+}Vβ3^{+} T cells by day 3 after immunization (Fig. 3A). This subpopulation grew in frequency through day 9 (Fig. 3B), reaching maximal total numbers by day 7 (Fig. 3C). These kinetics of CXCR5 expression are in close accord with the

kinetics of Vα11Vβ3-expressing T cell accumulation in follicular mantle zones and GCs during the response to PCC (9, 17). A subpopulation of the responding $CD44^+$ Vα11Vβ3-expressing T cells did not upregulate CXCR5 (Fig. 3). Such bimodality was not observed in the response of the monoclonal DO11.10 T cells to OVA-peptide (Fig. 1D) and may indicate that T cells with differing affinity for peptide/MHC differ in their propensity to upregulate CXCR5.

Increased responsiveness of CXCR5-expressing T cells to the follicular chemokine BLC

The rapid upregulation of CXCR5 on T cells following injection of antigen in adjuvant and the migration of a fraction of the cells into follicles suggested these cells had acquired responsiveness to BLC. This was tested directly *ex vivo* in transwell migration assays. OVA-specific T cells from draining LNs of adoptive transfer recipients immunized seven days earlier with OVA peptide s.c. in CFA showed a strong dose dependent response to BLC (Fig. 4A). By contrast, OVA-specific cells from recipients given the antigen i.v. in the absence of adjuvant did not respond to BLC (Fig. 4A). Kinetic analysis using cells from mice immunized with OVA peptide in CFA showed the BLC response was detectable by day 2 and was maximal between day 5 and day 10 (Fig 4B). In addition, flow cytometric analysis of transmigrated OVA-specific cells on day three revealed that while most input cells expressed CXCR5 (Fig. 1C), there was an enrichment for cells expressing the highest levels of CXCR5 in the responding population (Fig. 4C). These findings provide evidence for a direct relationship between

CXCR5 expression on T cells and acquisition of BLC responsiveness, although they do not establish whether the activation state of the cells also affects their ability to respond. To investigate the BLC responsiveness of CXCR5-expressing T cells with a resting phenotype we took advantage of previous observations that a subset of memory CD4 T cells express CXCR5 (20, 21). CD4 T cells from aged (≥1 year) mice were characterized as a source of memory phenotype T cells, and a great majority of the L-selectin^{lo} CD4 T cells from these animals were found to express CXCR5 (Fig. 4D). In addition to low L-selectin expression, the majority of CXCR5^{hi} T cells in young and old mice expressed high levels of CD44 and reduced amounts of CD45RB (data not shown). Most of the CXCR5^{hi} cells were also negative for the activation markers CD69 and CD25, further supporting their designation as memory cells. Interestingly, though a significant proportion of memory phenotype T cells in young mice expresses CXCR5, this proportion was consistently increased in aged mice (Fig. 4D). In *in vitro* chemotaxis assays the CXCR5^{hi} memory T cells showed a very similar dose sensitive BLC response to the activated OVA-specific T cells characterized above (Fig. 4E). These results establish that both resting and activated CXCR5^{hi} T cells respond to BLC. Interestingly, although the magnitude of the T cell response to BLC was lower than that observed for B cells, the T cells responded maximally to lower concentrations of BLC than did B cells (Fig. 4E). This finding is similar to that made previously with transfected Jurkat T cells (18), suggesting that T cells are intrinsically more sensitive than recirculating B cells to CXCR5 signaling. CD4 T cells express several-fold less surface CXCR5 than B cells (Fig. 1),

demonstrating that higher surface chemokine receptor expression does not equate to higher chemokine sensitivity.

CXCR5-expressing T cells have reduced responsiveness to the T zone chemokines ELC and SLC

T cells have been shown to respond strongly to ELC and SLC in *in vitro* chemotaxis assays (22-24, 27-30). Since these chemokines are expressed in the T zone and might be able to counteract the ability of a cell to respond to a chemokine made in follicles, we tested whether CXCR5-expressing T cells were altered in their responsiveness to ELC and SLC. In striking contrast to the elevated ELC and SLC responsiveness of *in vitro* activated T cells (28-30), OVA-specific T cells activated *in vivo* by subcutaneous peptide/CFA injection showed a significant down-regulation in responsiveness to these T zone chemokines (Fig. 5A). The time course of this decreased responsiveness to ELC (Fig. 5B) was similar to the time course over which the cells became responsive to BLC (Fig. 4B). In contrast, OVA-specific T cells from mice immunized i.v. with peptide in saline maintained their ability to respond to ELC throughout the ten day time course (Fig. 5B). Responsiveness to SDF1 was also decreased in the *in vivo* activated T cells (Fig. 5A), consistent with the recent *in vitro* finding that anti-CD3 stimulation reduces responsiveness to SDF1 (38). CXCR5-expressing memory phenotype cells from aged mice showed similarly low responsiveness to ELC and SLC (Fig. 5C). Interestingly, in these cells the responsiveness to SDF1 was elevated (Fig. 5C).

T cells can acquire the intrinsic ability to migrate to regions of lymphoid tissues proximal to follicles

The studies above demonstrate a tight relationship between CD4 T cell upregulation of CXCR5 expression, acquisition of BLC responsiveness, and migration into lymphoid follicles during an immune response. However, they do not establish whether intrinsic changes in the T cell are sufficient to direct these cells to follicles or whether additional (extrinsic) changes that accompany the adjuvant induced immune response are also needed. Although many of the CD4 T cells in aged mice express CXCR5 (see Fig. 4C), immunohistochemical analysis did not reveal a significantly greater number of T cells in follicles in aged mice compared to young mice (data not shown). When $CXCR5^{hi}$ CD4 cells were transferred from aged to young mice they were found to localize within the T zone, with only occasional cells migrating into follicles (Fig. 6A). These findings indicate that expression of CXCR5 is not sufficient to direct all types of T cells into B cell follicles. However, in contrast to aged normal mice, aged MRL-lpr mice contain very large numbers of T cells in a follicular distribution (39, 40). These CD3+ T cells are unusual in lacking CD4 and CD8 and in expressing B220 (39, 40). Flow cytometric analysis showed that they also express high surface CXCR5 (Fig. 7A) and in *in vitro* chemotaxis assays they demonstrated a robust response to BLC (Fig. 7B), and a reduced response to ELC and SLC (Fig. 7C). Importantly, the BLC dose response curve of the DN T cells was typical of $CXCR5^{+}$ CD4 T cells (compare Fig. 7B and Figs. 4A and D) and not of B cells

(see Fig. 4D). Although the follicular location of the DN T cells in MRL-lpr mice suggests these cells have acquired the intrinsic ability to migrate to follicles, MRL-lpr mice have multiple immunological abnormalities, and it was possible that homing of DN T cells to follicles was dependent on extrinsic changes in the lymphoid tissues. To test this directly, DN T cells were purified from LNs of MRL-lpr mice, labeled with CFSE, and transferred to normal syngeneic MRL mice. Strikingly, the transferred T cells migrated to regions proximal to B cell areas in all secondary lymphoid tissues of the recipient mice (Fig. 6). Differences in the distribution of the cells were noted in the different tissues. In the spleen, the T cells homed to the outer rim of the follicles, especially near the marginal zone bridging channels, and often the cells appeared in contact with marginal metallophilic macrophages (Fig. 6B). In LNs, the T cells homed to perifollicular and interfollicular locations (Fig. 6C), and in Peyer's patches the cells were seen to circle the whole follicular area (Fig. 6D). These observations demonstrate that T cells can acquire the intrinsic ability to migrate to the boundaries of lymphoid follicles.

Discussion

The findings above establish that immunization with antigen in adjuvant causes antigen-specific T cells to upregulate CXCR5 expression and acquire responsiveness to the follicular chemokine, BLC, while simultaneously becoming less responsive to the T zone chemokines, ELC and SLC. We propose that this reprogramming of responsiveness to B and T zone chemokines is part of the

mechanism by which antigen-activated T cells home to follicles to help initiate T-dependent antibody responses.

In the adoptive transfer studies of Jenkins and coworkers, it was observed that antigen needed to be injected in adjuvant for activated T cells to migrate to follicles (13, 34). When antigen was injected in the absence of adjuvant, T cell activation was transient and the activated cells failed to home to follicles. Our results provide a basis for understanding the different trafficking patterns of the activated cells as they show that CXCR5 upregulation and acquisition of BLC responsiveness only occurs following injection of antigen in adjuvant. Many studies have indicated that the effectiveness of adjuvants is through their potent activation of dendritic cells (41), and it is therefore reasonable to suggest that effective induction of CXCR5 expression on T cells requires interaction with appropriately activated antigen-presenting DC within the lymphoid tissue. OX40L is expressed by a subset of activated DC (42) and recent studies by Lane and coworkers provide evidence that stimulation of T cells through OX40 can promote upregulation of CXCR5 (43) and homing of T cells to follicles (44). Further studies are needed to define whether additional costimulatory molecules can regulate CXCR5 expression on T cells.

In vivo activation by antigen in adjuvant decreases T cell responsiveness to ELC and SLC at the same time as increasing responsiveness to BLC. This contrasts with findings *in vitro*, where PHA and IL-2 activated T cells responded more strongly than unactivated cells to SLC and ELC (28-30) and again indicates that the mode of T cell activation can strongly influence chemokine

responsiveness. Recently it has been shown that *plt/plt* mice, which exhibit defective homing of T cells to splenic T zones and LNs (45), have a compound defect that causes a deficiency in SLC expression and markedly reduced ELC expression (31). This finding provided strong evidence that SLC and ELC are needed for T cell homing to lymphoid T cell areas. Reduced responsiveness of $CXCR5^{hi}$ T cells to ELC and SLC may therefore allow the cells to more readily leave the T zone and enter follicles. Reciprocally, the failure of T cells activated following intravenous injection of antigen to downregulate their SLC and ELC response might contribute to their inability to migrate to follicles. Since SLC appears important for cells to enter LNs via HEV or lymphatics (22, 31, 46), decreased responsiveness to this chemokine is also likely to influence the recirculation pattern of the cells.

Our studies provide strong evidence that altered responsiveness to constitutively expressed chemokines is part of the mechanism by which antigen-activated CD4 T cells migrate towards and into B cell follicles. This conclusion is also supported by the transfer experiments showing that DN T cells from MRL-lpr mice are intrinsically capable of migrating to areas proximal to follicles (Figure 6). However, the failure of $CXCR5^{hi}$ CD4 T cells to migrate to follicles following adoptive transfer suggests that additional factors might normally help guide antigen-activated CD4 T cells. The migration of only a subset of OVA-activated T cells to follicles also suggests that $CXCR5^{hi}$ cells may be heterogeneous in their responsiveness to these factors. Several studies have shown that BCR-stimulated B cells upregulate expression of chemokines, including MIP1α, MIP1β

(47, 48) and MDC (49) that can attract subsets of activated T cells (50-53). Since antigen-activated B cells move to the boundary of B and T zones (3), it is likely that chemokines produced by activated B cells work together with constitutively expressed chemokines to bring antigen-activated T cells to the outer T zone. Whether further cues are needed to direct cells from the outer T zone into follicles remains unclear, although the failure of MRL-lpr DN T cells to migrate to the inner regions of follicles suggests this is the case. Possibilities include changes in the responsiveness of the T cells to other unknown chemokines, and changes in the relative adhesiveness of the cells for features of the B or T cell compartments. Several examples of cell sorting occurring as a result of differential adhesiveness of cells have been reported (54-56).

A major role of T cells inside follicles is to support the GC response (1, 2). Nine days after immunization with PCC in adjuvant, a large majority of the PCC-responsive Vα11Vβ3-expressing T cells express CXCR5 (Fig. 3) and at this time point, approximately 75% of the cells are localized within GCs (17) . Therefore, at least a subset of PCC-responsive $CXCR5^{hi}$ T cells acquire the ability to enter GCs. Similarly, in the DO11.10 adoptive transfer system, ten days after immunization with OVA peptide in CFA the majority of $KJ1\text{-}26^{+}$ T cells were $CXCR5^{hi}$, and many cells were found in follicular mantle zones and GCs (Fig. 2D). The strong expression of BLC in primary follicles (18) and in follicular mantle zones of secondary follicles (Fig. 2E, F) is consistent with BLC playing a role in attracting T cells to these sites. The presence of only small numbers of BLC expressing cells within GCs, predominantly in the region distal to the T zone

(most likely corresponding to the GC light zone (1)) suggests that while BLC/CXCR5 might have a role in helping position cells within GC, additional cues are likely to be needed. These findings also establish that there is substantial heterogeneity amongst follicular stromal cells in terms of BLC expression levels, with GC follicular dendritic cells expressing relatively little of this chemokine. Perhaps by being concentrated predominantly outside the T zone-distal pole of the GC, BLC helps polarize the GC light and dark zone compartments. The notion that cues other than BLC play important roles in GC organization is supported by the finding that GCs are able to form in CXCR5-deficient mice (21). Furthermore, although spleens of CXCR5-deficient mice lack polarized follicles and contain aberrantly located GCs, the follicular disruption in LNs appeared minimal (21). While this suggests that the role of CXCR5/BLC in B and T cell homing to LN follicles is redundant to other chemokine/receptor systems, studies in mice lacking lymphotoxin or TNF have shown that effects on follicular organization in LNs are more difficult to detect than in spleen or Peyer's patches (57, 58) yet these effects can still be substantial (59, 60). As we have shown here, BLC is expressed in LN follicles and CXCR5 is strongly upregulated on activated T cells in LNs. Future studies of BLC-deficient mice should help further dissect the contribution of BLC and CXCR5 to follicular organization and GC formation in LNs.

T cell homing to follicles may be important not only for providing help to B cells, but also for providing activated T cells with growth and survival signals. This possibility is suggested by the finding that antigen injected in the absence of

adjuvant fails to promote T cell migration to follicles, and also fails to promote survival of activated or memory T cells (13, 34). The selective accumulation of the CXCR5-expressing subset of CD4 T cells in aged mice (Fig. 4C) and in HIV-infected humans during disease progression (61) is consistent with the notion that trafficking through B cell areas plays a role in long-term survival of memory T cells. This possibility is also supported by the finding of a defect in CD4 T cell memory in B cell deficient mice (62). Although we did not observe trafficking of $CXCR5^{hi}$ memory T cells to follicles in short-term transfer experiments, studies in rats have suggested that memory T cells migrate through follicles at greater frequency than naïve T cells (63).

In summary, our findings suggest a model for how helper T cells home to follicles. Following engagement of peptide-MHC complexes on appropriately activated T zone DC, CD4 T cells upregulate CXCR5, acquire responsiveness to the follicular chemokine BLC and simultaneously down-regulate responsiveness to the T zone chemokines ELC and SLC. Together with additional presently undefined changes, this reprogrammed chemokine responsiveness helps propel T cells toward B cell areas. Further cues, such as those emanating from activated B cells and from GC cells can then act upon these T cells to more precisely control their positioning and facilitate their ability to act as B cell helpers.

Materials and Methods

Mice

Six-10 week old Balb/cAnN mice were obtained from Charles River Laboratories (Wilmington, MA). C57BL/6 (B6), MRL/MpJ (MRL), and MRL/Mp-*lpr/lpr* (MRL-lpr) mice were from Jackson Laboratories (Bar Harbor, ME). D011.10 TCR-transgenic mice (32) on the Balb/c background, and aged and control B6 mice for memory T cell analysis were maintained in the UCSF animal care facility. Eight-10 week old, specific pathogen-free, male B10.BR mice (Jackson Laboratories) were housed under barrier conditions at the Duke Vivarium.

Chemokines

HIS_6-tagged murine BLC was prepared by PCR-based insertion of 6 histidine codons preceding the BLC stop codon. The BLC-his6 construct was inserted into the CMV-based mammalian expression vector pRK5 (33) and stably transfected into HEK-293 cells using the Lipotaxi Mammalian Transfection Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. HIS_6-BLC was purified from tissue culture supernatants using a NiNTA column (Qiagen Inc., Valencia, CA). The protein was eluted in 100μM imidazole (Fisher Scientific Company, Pittsburgh, PA). SDS-PAGE separation of the eluate revealed a band representing >90% of total protein corresponding to the recombinant HIS_6-BLC. HIS_6-ELC was produced in bacteria and purified as described (23). A similarly constructed vector for bacterial HIS_6-SLC production was a gift from M. Gunn.

Stromal-cell derived factor (SDF)1α (N33A) produced by chemical ligation (Gryphon Sciences, South San Francisco, CA), HIS_6-BLC, -ELC, and -SLC were used in all chemotaxis assays, except for MRL-lpr chemotaxis where, due to availability at the time of the experiments, non-HIS-tagged murine SLC (gift of M. Gunn) and human ELC (R&D Systems, Minneapolis, MN) were used. We have not observed significant differences in the response of mouse cells to mouse or human ELC.

DO11.10 T cell adoptive transfer, OVA immunization, and recipient analysis

Adoptive transfer and immunization of recipients was carried out essentially as described (13). Lymphocytes were isolated from LNs or spleen of DO11.10 donor mice and the percentage of $OVA_{323-339}$ peptide/I-A^d-specific $CD4^+$ T cells was determined by flow cytometric analysis of an aliquot of cells stained with FITC-conjugated clonotypic mAb KJ1-26 and anti-CD4-PE (Caltag Laboratories Inc., Burlingame, CA). 2.5×10^6 KJ1-26^+CD4^+ cells were adoptively transferred into sex-matched Balb/c recipients by i.v. injection. The day following cell transfer, mice were immunized with 300μg $OVA_{323-339}$ peptide either emulsified in CFA (Sigma Chemical Co., St. Louis, MO) and injected s.c. in a total volume of 0.1 mL distributed over 3 points on the back, or in sterile PBS by i.v. injection. Recipients were sacrificed and dissected 2, 3, 5, 7, 10, 14, or 25 days after immunization. In a second immunization protocol (34) mice were injected s.c. with 2 mg OVA protein (Sigma) mixed in 0.2 ml of 250 μg/ml LPS (Sigma). For s.c. injected mice, lymphocytes were isolated from axillary and

brachial LNs. For i.v. injected mice, cells from mandibular, cervical, axillary, brachial, inguinal, and in some cases mesenteric nodes were pooled. The remaining peripheral LNs were frozen in OCT (Miles, Elkhart, IN) for sectioning. Flow cytometric analysis was performed using affinity-purified anti-CXCR5 rabbit antiserum (35), followed by biotinylated goat anti-rabbit IgG (Pharmingen, San Diego, CA) with normal mouse and rat serum (1/100 dilution), and then streptavidin-cychrome (Pharmingen), KJ1-26-FITC, anti-B220-PE and anti-CD8-PE (Caltag).

PCC immunization and flow cytometry

Whole pigeon cytochrome c (PCC)(Sigma, St. Louis, MO) was diluted into PBS and mixed with the Ribi adjuvant system (RAS)(Ribi Immunochem Research, Hamilton, MT). B10.BR mice were immunized with 400µg of PCC in 200µl of adjuvant emulsion in two 100µl doses by s.c. injection on either side of the base of tail. Animals were sacrificed at 3, 5, 7, and 9 days after immunization and the draining LNs harvested as previously described (17). In brief, inguinal and periaortic nodes were collected and using 0.17M NH_4Cl solution for erythrocyte lysis made into single cell suspensions. Cells were incubated with anti-CXCR5 rabbit antiserum, followed by anti-rabbit IgG-biotin (Santa Cruz Biotechnology, Santa Cruz, CA). After blocking with normal rabbit and mouse serum (1/100 dilution) for 5 minutes, staining was completed using streptavidin-PE (Pharmingen), anti-Vα11-FITC (Pharmingen), anti-Vβ3-allophycocyanin, anti-B220-Cy5PE (Pharmingen), anti-CD8-Cy5PE (Pharmingen), anti-CD11b- Cy5PE

(Caltag), and anti-CD44-Texas Red. Finally, cells were resuspended in 2μg/mL propidium iodide (PI; for dead cell exclusion) in PBS with 5% FCS. The cells were analyzed using a dual laser modified FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA) (an argon laser as the primary, a tunable dye laser as the secondary) capable of seven parameter collection. Files were acquired using CellQuest software (Becton Dickinson) and analyzed using FlowJo software (Tree Star Inc.; San Carlos, CA).

Chemotaxis Assays

Chemotaxis assays were performed as described (23) using 10^6 total cells per 5 μm transwell (Corning Costar Corp., Acton, MA). To identify migrating populations, a fraction of transmigrated cells were stained and analyzed by flow cytometry. Transmigrated LN cells from Balb/c recipients of OVA-specific T cells were stained with KJ1-26-FITC and anti-CD4-TriColor (Caltag), or with anti-CD4-PE (Caltag) and KJ1-26-biotin followed by streptavidin-cychrome (Pharmingen). Because BLC causes reversible internalization of CXCR5 (36), transmigrated splenocytes from aged B6 mice were washed twice and incubated in RPMI+0.5% BSA for one hour at 37°C 5% CO_2 to allow CXCR5 re-expression before staining with anti-CXCR5 rabbit antiserum/goat anti-rabbit IgG-biotin/streptavidin-cychrome, anti-CD4-FITC (Caltag), and anti-CD62L-PE (Pharmingen). LN suspensions from 5-8 month-aged MRL-lpr mice were stained with anti-B220-PE, anti-Thy1.2-biotin (Caltag), and anti-CXCR5 rabbit antiserum followed by goat anti-rabbit IgG-FITC (Caltag) and streptavidin-cychrome. To

provide an internal control, MRL-lpr splenocytes (70% $Thy1^+B220^+$, 9% $Thy1^+B220^-$) were mixed 3:1 with B6 splenocytes (<1% $Thy1^+B220^+$, 27% $Thy1^+B220^-$) for chemotaxis assays. Transmigrated cells were stained with anti-Thy1.2-FITC (Caltag), anti-B220-PE, and anti-CD21-biotin/streptavidin-cychrome.

MRL-lpr DN T cell and aged B6 $CXCR5^{hi}$ T cell adoptive transfers

DN T cells were purified from LNs of 5 to 8 month old MRL-lpr mice. Total LN cells were incubated with biotinylated mAbs against CD22 (Pharmingen), CD4 and CD8 (Caltag) followed by streptavidin-coated magnetic beads, and then passed over a MACS® column (Miltenyi Biotec, Inc., Auburn, CA). Memory phenotype splenocytes from 14 months-aged mice were enriched by MACS® depletion with biotinylated mAb against CD8, B220, and CD11b (Caltag). For all transfers, 2×10^7 cells were labeled with 5- (and 6-) carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) as described (35) and transferred by i.v. injection into appropriate syngeneic (MRL or B6) recipients. After approximately 24 hours, recipients were sacrificed and spleen, LNs, and Peyer's patches frozen in OCT for sectioning.

Immunohistochemistry, Immunofluorescence Microscopy and In situ Hybridization

For immunohistochemistry, cryostat sections (7-8μm) were fixed and stained as previously described (35) with the following reagents: biotinylated or

FITC-conjugated KJ1-26, rat anti-CD4 and anti-CD8 (Caltag), and biotinylated peanut agglutinin (Sigma). Biotinylated reagents were detected with avidin-alkaline phosphatase (Sigma), rat mAbs with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (Southern Biotechnology Associates), and KJ1-26-FITC with HRP-conjugated anti-fluorescein (NEN, Boston, MA). Enzyme reactions were developed with conventional substrates for peroxidases (diaminobenzidine/H_2O_2; Sigma) and alkaline phosphatase (FAST RED/Naphthol AS-MX; Sigma). Endogenous alkaline phosphatase activity was blocked with levamisole (Sigma). Some sections were counterstained with hematoxylin (Fisher Scientific Co.). Sections were mounted in crystal mount (Biomeda Corp., Foster City, CA). For immunofluorescence microscopy, unfixed sections were air-dried and incubated with biotinylated mAbs against CD8, CD4, or Thy1.2 (Caltag) and CD3ε (Pharmingen) followed by streptavidin-Cy3 (Jackson Immunoresearch, West Grove, PA). Three color staining of spleen sections was achieved by costaining with rat anti-MOMA-1 (37) followed by goat anti-rat IgG-aminomethylcoumarin (Jackson Immunoresearch). Sections were mounted in Fluoromount G (Southern Biotechnology Associates, Inc., Birmingham, AL), viewed and photographed as described (35). In situ hybridization analysis was performed as described (23) using a BLC probe spanning nucleotides 27 to 1042 of mouse BLC (18).

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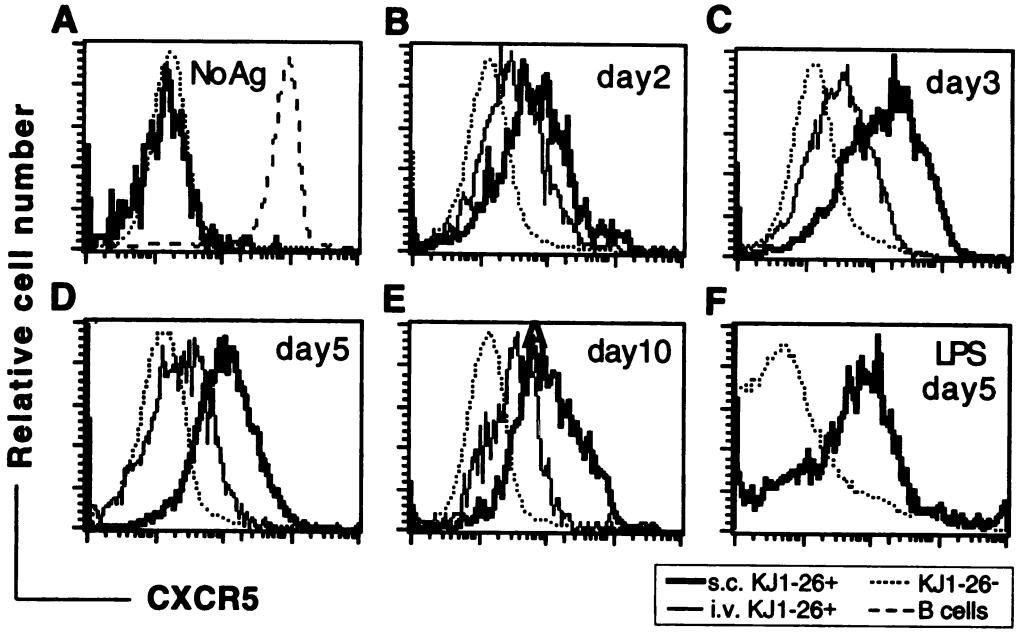
Figure 1

CXCR5 upregulation by *in vivo*-activated OVA-specific T cells.

(A-E) Histograms depict CXCR5 expression on non-OVA-specific CD4 cells *(KJ1-26$^-$)* and on gated KJ1-26$^+$B220$^-$CD8$^-$ cells from mice that were treated as follows: *(A)* unimmunized; *(B-E)* immunized with OVA peptide s.c. in CFA *(s.c. KJ1-26$^+$)* or i.v. in saline *(i.v. KJ1-26$^+$)*; *(F)* immunized with OVA protein in LPS. In *A* the level of CXCR5 expression on B cells is shown for comparison (dashed line). In *B-F* the number of days elapsed between immunization and analysis is displayed in each panel. No Ag: No antigen injected. The experiment shown in *(F)* was performed at a different time to *(A-E)* and has a different level of background staining. Results are representative of at least three independent experiments at each time point except panel *(F)* which is representative of two experiments.


A
NoAg
B
day2
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F
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Relative cell number
CXCR5
s.c. KJ1-26+
i.v. KJ1-26+
KJ1-26-
B cells

Figure 2

Follicular homing of *in vivo*-activated OVA-specific T cells and expression pattern of BLC in secondary follicles.

(A-C) Staining of LN sections to detect KJ1-26$^+$ OVA-specific T cells in red and CD4 and CD8 in brown. *(D)* Staining with peanut agglutinin (PNA) to detect GCs in red, and with KJ1-26 to detect OVA-specific T cells in brown. Sections are from draining LNs of mice that received OVA-specific CD4 T cells and were immunized with OVA peptide s.c. in CFA three *(A)*, five *(B)* or ten *(D)* days before isolation, or i.v. in saline *(C)* three days before isolation. *(E* and *F)* In situ hybridization analysis of BLC expression pattern in LNs containing secondary follicles. BLC hybridization is seen as dark blue staining and sections are contained in brown for B220 *(E)* or PNA *(F).* The capsular staining in *E* was also seen in controls and is nonspecific. FO: follicle, T: T cell zone, FM: follicular mantle region, GC: germinal center. Original magnification: *A, C, E and F,* ×10 objective; *B* and *D,* ×5 objective.

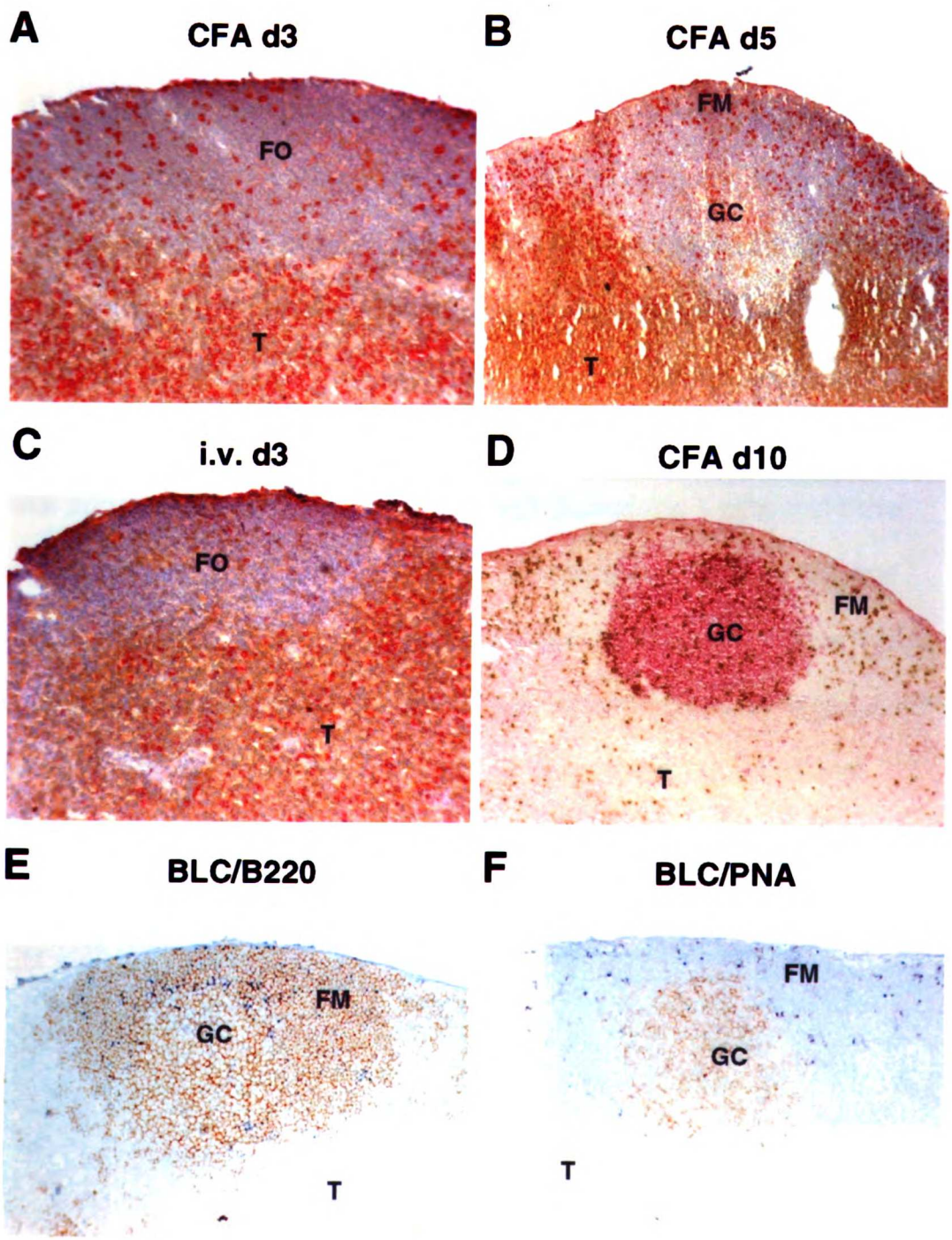

A
CFA d3
FO
T
B
CFA d5
FM
GC
T
C
i.v. d3
FO
T
D
CFA d10
GC
FM
T
E
BLC/B220
FM
GC
T
F
BLC/PNA
FM
GC
T

Figure 3

Antigen-specific CD4 T cell expression of CXCR5 during a primary immune response.

(A) Representative probability contours for CD44 and CXCR5 expression on Vα11Vβ3-expressing T cells over the course of a primary response. All profiles are presented as 5% probability contours with outliers and are PI^- $CD8^-$ $B220^-$ $CD11b^-$ $V\alpha11^+$ $V\beta3^+$. The day after antigen administration is displayed above each panel. The quadrants are defined by the cross hairs and indicate the limits of CD44 and CXCR5 regulation that were used to calculate the frequencies of cellular subsets. *(B)* Frequencies of Vα11Vβ3-expressing T cells that have upregulated CD44 and are either $CXCR5^-$ *(first panel)* or $CXCR5^+$ *(second panel)*. *(C)* The total number of antigen-activated Vα11Vβ3-expressing T cells in the draining LNs as calculated using the frequencies obtained by flow cytometry and total cell counts estimated when organs were harvested; panel one represents the $CXCR5^-$ subset, panel two represents the $CXCR5^+$ subset. Each estimation *(B and C)* is presented as the mean from at least 3 separate animals ±SEM.

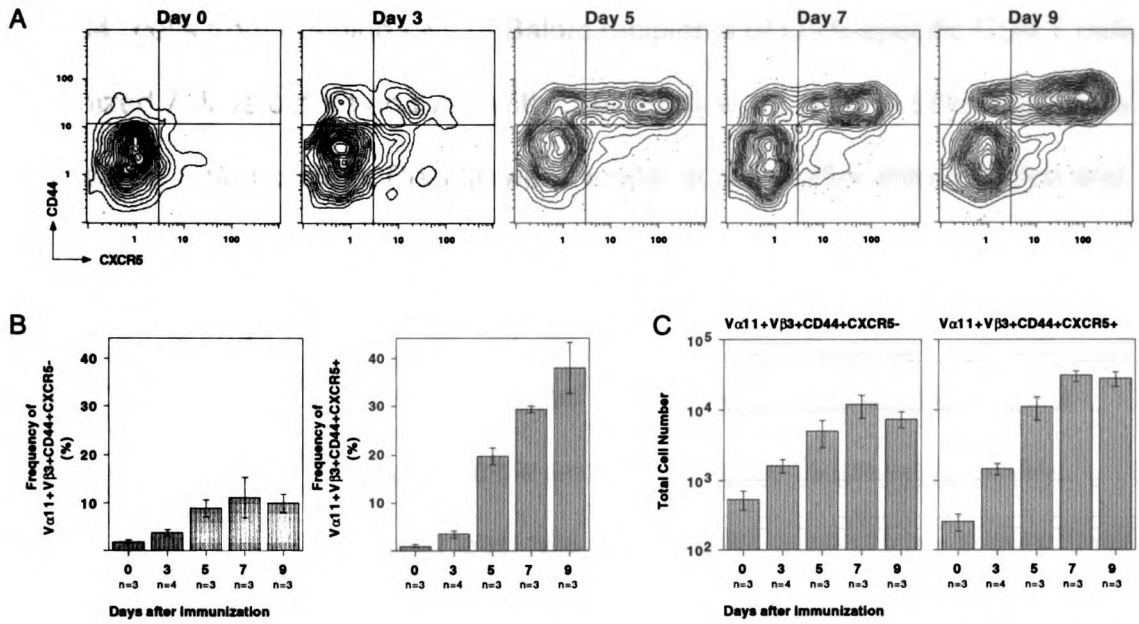

A
Day 0
Day 3
Day 5
Day 7
Day 9
CD44
CXCR5
B
Frequency of Vα11+Vβ3+CD44+CXCR5- (%)
Frequency of Vα11+Vβ3+CD44+CXCR5+ (%)
Days after immunization
C
Vα11+Vβ3+CD44+CXCR5-
Vα11+Vβ3+CD44+CXCR5+
Total Cell Number
Days after immunization

Figure 4

BLC chemotactic response of *in vivo*-activated OVA-specific T cells and memory phenotype CXCR5-expressing T cells.

Results are expressed as percentage of input cells transmigrated. Lines represent means of duplicate transwells. *(A)* Chemotactic response of KJ1-$26^{+}CD4^{+}$ cells from draining LNs of Balb/c recipients of OVA-specific CD4 T cells immunized 7 days before analysis with OVA peptide s.c. in CFA (filled circles) or i.v. in saline (open circles). Results were similar at day 5 after immunization and are representative of five independent experiments. *(B)* Kinetic analysis of OVA-specific T cell acquisition of BLC responsiveness. The day after immunization with OVA peptide s.c. in CFA is indicated in each panel. Differences in basal migration levels were not reproducible and reflect assay to assay variability. Results are representative of at least two independent experiments at each time point.*(C)* CXCR5-expression on KJ1-26^{+} $CD4^{+}$ cells from day 3 draining LNs showing the total input population (input) and the cells that migrated to BLC (migrated). *(D)* CXCR5 and L-selectin (CD62L) expression on $CD4^{+}$ splenocytes from young *(left panel)* and 21 month-aged *(right panel)* B6 mice. Numbers represent the percentage of $CD4^{+}$ and lymphocyte size gated cells in each quadrant. Similar results, with progressive accumulation of L-$selectin^{lo}$ and $CXCR5^{hi}$ T cells were obtained for more than 10 animals under three or over twelve months of age. *(E)* Chemotactic response of $CXCR5^{hi}$ (filled diamonds) and $CXCR5^{lo/-}$ (open diamonds) $CD4^{+}$ T cells, and of B cells (squares) from the

spleen of a 21 month-aged mouse. The y-axis on the left refers to T cells and on the right to B cells. Results are representative of eight independent experiments.

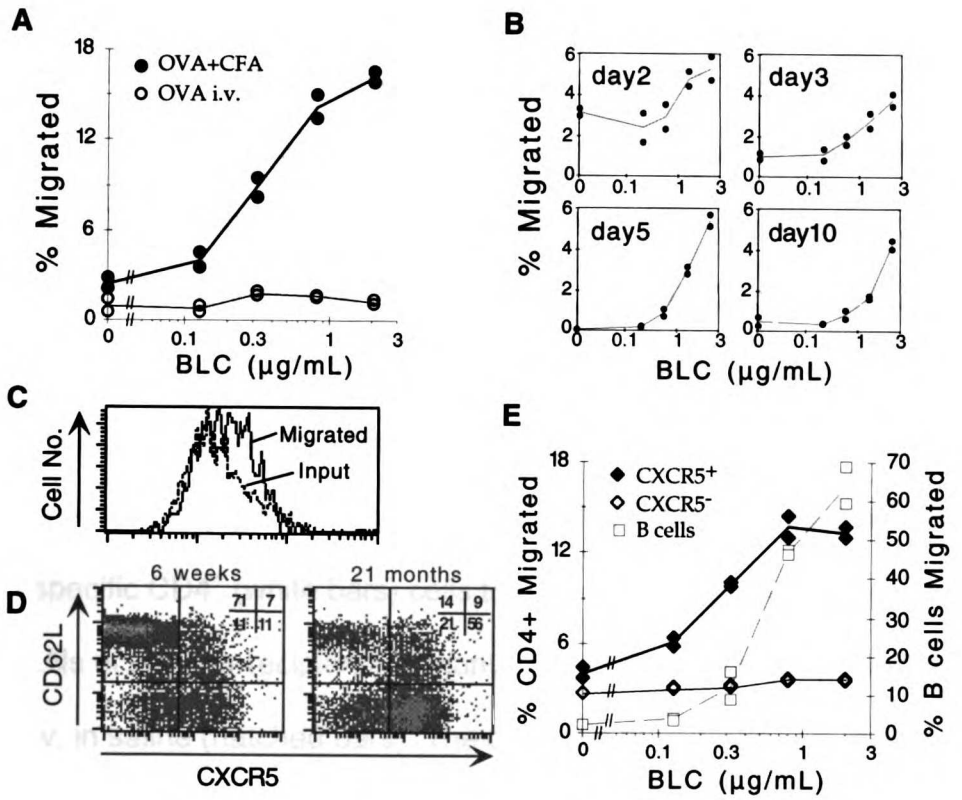

A
% Migrated
OVA+CFA
OVA i.v.
BLC (µg/mL)
B
day2
day3
day5
day10
% Migrated
BLC (µg/mL)
C
Cell No.
Migrated
Input
D
6 weeks
21 months
CD62L
CXCR5
E
CXCR5+
CXCR5-
B cells
% CD4+ Migrated
% B cells Migrated
BLC (µg/mL)

Figure 5

Chemotactic response profiles of *in vivo*-activated OVA-specific T cells and memory phenotype CXCR5-expressing T cells.

Results are expressed as percent of input cells transmigrated. Bars represent means of duplicate transwells. *(A)* Chemotaxis of KJ1-26$^+$CD4$^+$ OVA-specific (shaded bars) and non-specific CD4$^+$ (white bars) cells from draining LNs of OVA-specific T cell transfer recipients immunized 7 days previously with OVA peptide s.c. in CFA. Chemokine concentrations were: BLC, 2μg/mL; ELC, 0.2μg/mL; SLC, 0.2μg/mL; SDF1, 0.3μg/mL. Results were similar at day 5 after immunization and are representative of at least three independent experiments for each chemokine. *(B)* Response of KJ1-26$^+$CD4$^+$ OVA-specific (shaded bars) and non-specific CD4$^+$ (white bars) cells to 0.2 μg/mL ELC. Cells are from draining LNs of transfer recipients immunized with OVA peptide s.c. in CFA (solid bars) or i.v. in saline (hatched bars). The day after immunization is indicated on the x-axis. Data at day 5 are representative of five experiments for s.c. in CFA and two experiments for i.v. in saline. *(C)* Chemotaxis of CXCR5$^{lo/-}$ (white bars) and CXCR5^{hi} (shaded bars) CD4$^+$ T cells from the spleen of a 21 month-aged mouse. Chemokine concentrations were: BLC, 2 μg/mL; ELC, 0.5 μg/mL; SLC, 0.8μg/mL; SDF1, 0.3 μg/mL. Results are representative of at least four independent experiments for each chemokine. CXCR5^{hi} T cells exhibited reduced responsiveness to ELC (0.02 to 1.5μg/mL) and SLC (0.08 to 1.2μg/mL) at all chemokine concentrations tested.

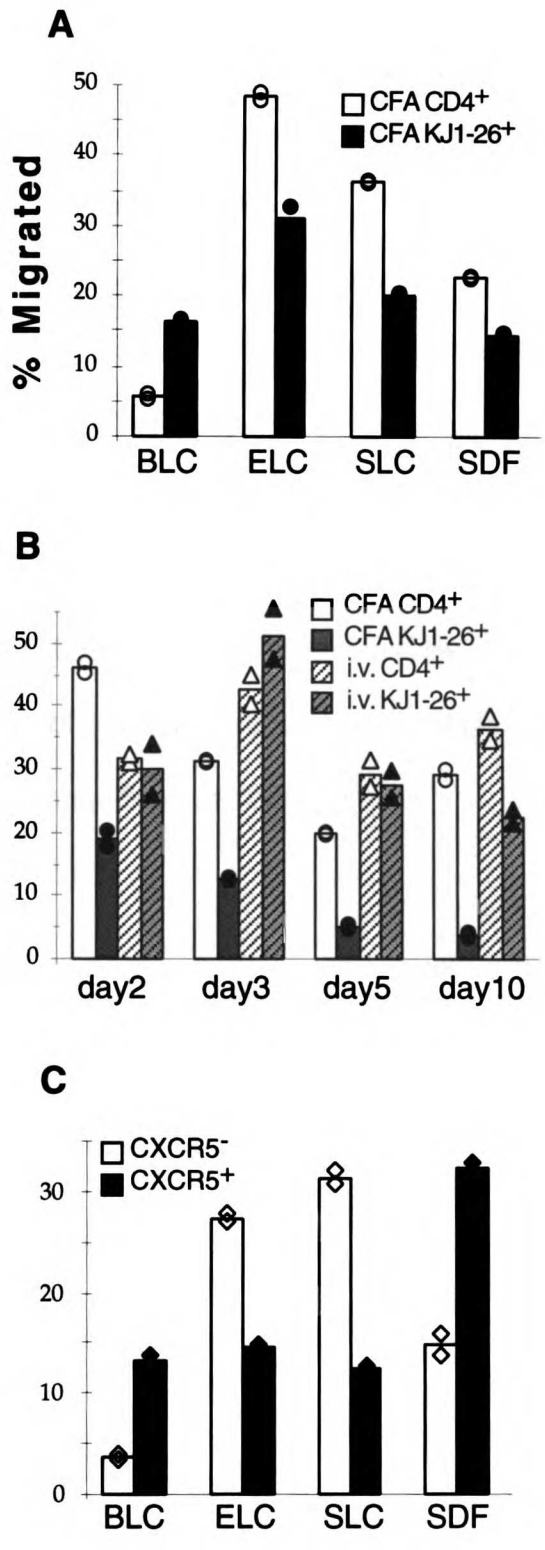

A
% Migrated
50
40
30
20
10
0
CFA CD4+
CFA KJ1-26+
BLC
ELC
SLC
SDF
B
CFA CD4+
CFA KJ1-26+
i.v. CD4+
i.v. KJ1-26+
day2
day3
day5
day10
C
CXCR5-
CXCR5+
30
20
10
0
BLC
ELC
SLC
SDF

Figure 6

Transferred MRL-lpr DN T cells, but not memory phenotype CXCR5^{hi} T cells home towards B cell follicles in secondary lymphoid organs of unimmunized non-autoimmune mice.

Spleen *(A-B)*, LN *(C)*, and Peyer's patch *(D)* sections from syngeneic recipients of CFSE-labeled *(A)* CD4^{+}CXCR5^{+}-enriched splenocytes from 12 to 15 month-aged mice or *(B-D)* purified DN T cells from 8 month-aged MRL-lpr mice. Recipient tissues were isolated one day after cell transfer. CFSE labeled cells are green. T cells are stained in red using mAbs to CD3ε and Thy1.2 *(A-B)*, CD8 *(C)*, or CD4 *(D)*. MOMA-1^{+} marginal zone metallophilic macrophages are stained blue. *A* is representative of 5 and *B-D* of 3 mice. F: follicle, T: T cell zone, MZ: marginal zone. Original magnification: ×10 objective.

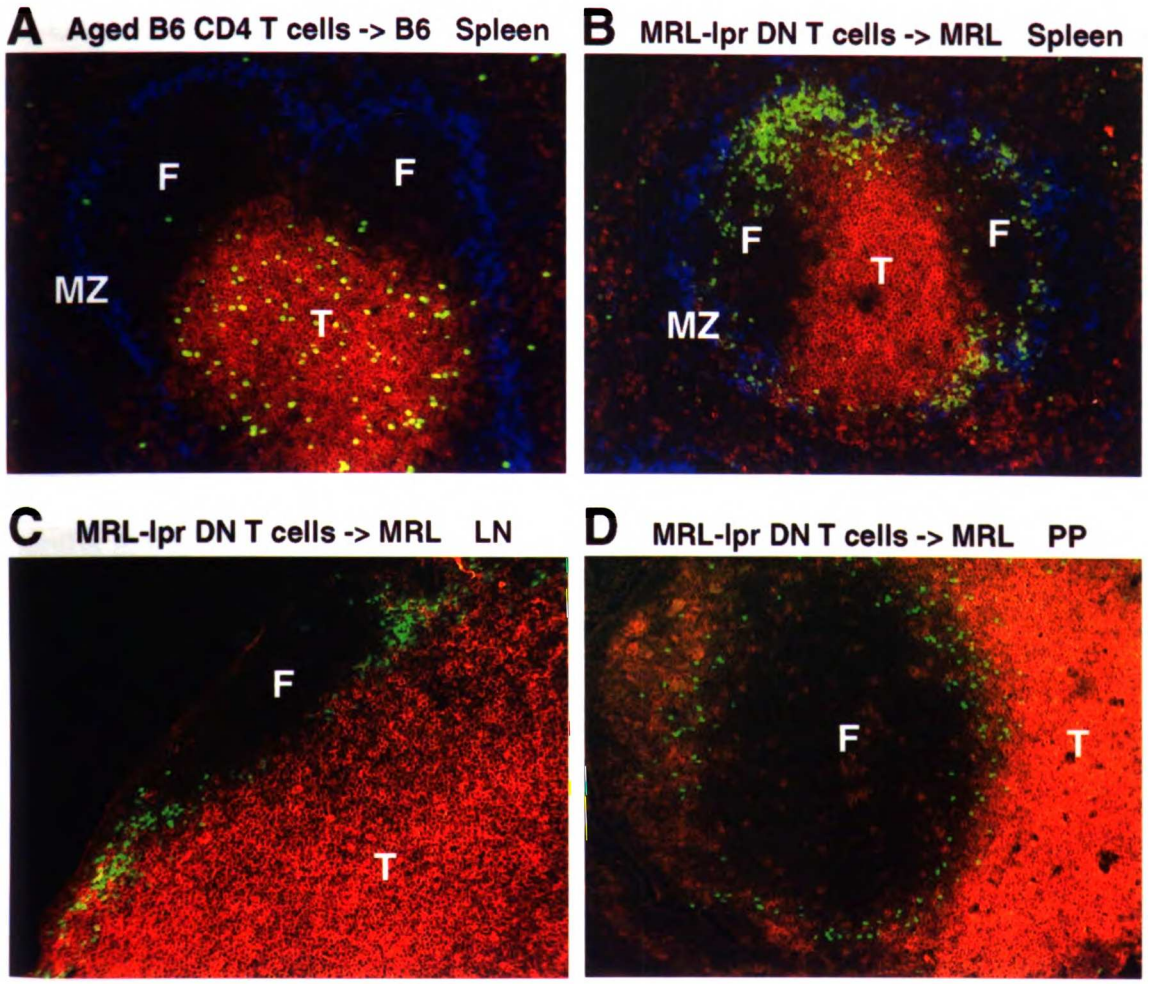
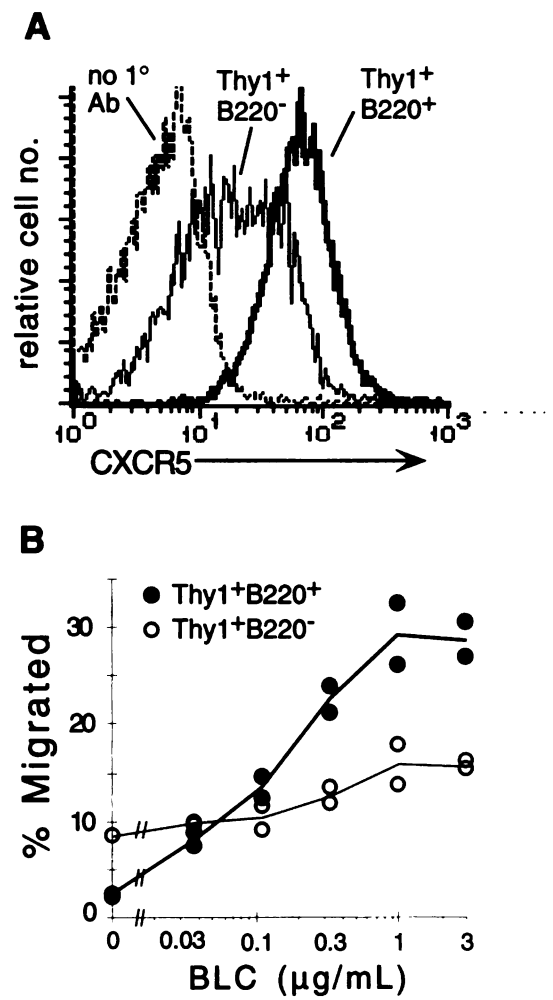

A Aged B6 CD4 T cells -> B6 Spleen
F
F
MZ
T
B MRL-lpr DN T cells -> MRL Spleen
F
F
T
MZ
C MRL-lpr DN T cells -> MRL LN
F
T
D MRL-lpr DN T cells -> MRL PP
F
T

Figure 7

CXCR5 expression and chemotactic response profile of DN T cells from MRL-lpr mice.

(A) CXCR5 expression on DN *($Thy1^+B220^+$)* and conventional *($Thy1^+B220^-$)* T cells. DN T cells stained with the secondary antibody alone *(No 1° Ab)* are shown as a control. (*B* and *C*) Chemotaxis of a 3:1 mixture of MRL-lpr and C57BL/6 splenocytes in response to *(B)* BLC and *(C)* a panel of lymphoid chemokines. Results are expressed as percent of input cells transmigrated for DN T cells (filled circles) and conventional T cells (open circles). Chemokine concentrations in *B*: BLC, ELC, and SLC, 1μg/mL; SDF1, 0.3μg/mL. MRL-lpr mice were aged 5 months at the time of analysis. Lines *(B)* and bars *(C)* represent means of duplicate transwells. Results in *(A)* are representative of three, and in *(B-C)* of two independent experiments.


A
no 1°
Ab
Thy1+
B220-
Thy1+
B220+
relative cell no.
10^0
10^1
10^2
10^3
CXCR5
B
Thy1+B220+
Thy1+B220-
% Migrated
30
20
10
0
0
0.03
0.1
0.3
1
3
BLC (µg/mL)
C
40
30
20
10
0
% Migrated
Thy1+B220+
Thy1+B220-
BLC
ELC
SLC
SDF

Chapter 4

CXCL13 is required for B1 cell homing, natural antibody production and body cavity immunity

Summary

B1 cells are a predominant cell type in body cavities and an important source of natural antibody. Here we report that in mice lacking the chemokine, CXCL13, B1 cells are deficient in peritoneal and pleural cavities but not in spleen. CXCL13 is produced by cells in the omentum and by peritoneal macrophages, and in adoptive transfers, B1 cells home to the omentum and then the peritoneal cavity in a CXCL13-dependent manner. $CXCL13^{-/-}$ mice are deficient in pre-existing phosphorylcholine (PC)-specific antibodies, and in their ability to mount an anti-PC response to peritoneal streptococcal antigen. These findings provide new insight into the mechanism of B1 cell homing and establish a critical role for B1 cell compartmentalization in production of natural antibodies and for body cavity immunity.

Introduction

B1 cells are specialized B cells that are distinguished from the major recirculating B cell population (B2 cells) by their unique tissue distribution, distinct cell surface phenotype, capacity for self-renewal and prominent role in natural antibody production (1-4). B1 cells are present at a low frequency in the spleen, but are the predominant B cell population in the major body cavities, the peritoneal, pleural and pericardial cavities (1). B1 cells express high levels of surface IgM and low levels of IgD, CD23, and B220. In the body cavities, B1 cells can be distinguished from B2 cells by their expression of the integrin Mac-1. A major fraction of B1 cells express CD5 (formerly Ly1) and $CD5^+$ B1 cells are known as B1a cells, while the remaining $CD5^{lo/-}$ B1 cells are designated B1b (1, 2).

In wildtype mice, B1a cells develop predominantly from fetal hematopoietic stem cells, whereas B1b cells develop from both fetal and adult stem cells (1). The development of B1 cells is strongly influenced by antigen receptor specificity (2), and certain immunoglobulin (Ig) gene rearrangements are restricted to expression in B1 cells (2). Among the known B1-associated antigen receptors are several that react with autoantigens (5-7), and others that bind conserved epitopes present on common pathogens (8-10). One well-characterized example is the rearrangement of the germline $V_H1/\kappa22$ genes, which encode T15 idiotype-containing antibodies that bind phosphorylcholine (PC), a hapten present on the surface of many pathogenic bacteria (8, 10).

Although B1 cells constitute only a few percent of all B cells, they are a significant source of serum antibody (1, 11) and they make a dominant contribution to low affinity IgM antibodies that are present in serum of unimmunized mice, known as natural antibodies (12), including antibodies that bind PC (8) and phosphatidylcholine (9). Studies in mice deficient in natural antibodies have established their critical role in providing early protection from a variety of pathogens (13-16). An especially prominent role has been demonstrated for PC-binding antibodies in protection from bacterial infections (10, 17).

Despite the importance of the B1 cell population, relatively little is known about how the cells enter the body cavities, or what factors guide their homing. Early studies indicated that the B cells that could be released from the peritoneum by peritoneal lavage might be related to B cells present in the omentum (18). The omentum is a bilayered sheet of mesothelial cells connecting the spleen, pancreas, stomach and transverse colon, terminating in an apron-like structure that contains numerous adipocytes ((18), also pictured in Figure 5A). The omentum has long been appreciated for its role in abdominal wound repair and particle capture (18). Aggregates of macrophages and lymphocytes, and smaller numbers of mast cells and plasma cells, termed 'milky spots', have been described in both the thin mesothelial sheets and the fat pads of the omentum (18). Interestingly, the mesothelium is fenestrated at sites overlying milky spots, and cells have been observed to migrate between these structures and the surface of the mesothelium (18). However, to our knowledge the phenotype of

the B cells in the omentum has not been characterized and it is not known whether there is an interchange of B1 cells between the omentum and the peritoneal cavity.

Recently, chemokines and chemokine receptors have been shown to play a critical role in lymphocyte homing to subcompartments of lymphoid organs (19-21). B cell homing to lymphoid follicles is directed by the chemokine CXCL13 (formerly BLC or BCA1), a chemokine made in a lymphotoxin (LT)-dependent manner by follicular stromal cells (22, 23), and mice lacking CXCL13 or the CXCL13 receptor, CXCR5, fail to form lymphoid follicles (24, 25). Homing to T cell zones is similarly controlled by CCR7 in conjunction with its ligands, CCL19/ELC and CCL21/SLC (26, 27). The CXCR4 ligand, CXCL12/SDF-1, is also constitutively expressed in lymphoid organs, and it plays an important role in B cell development and plasma cell homing (28, 29).

Here we demonstrate that CXCL13 is constitutively expressed by cells in the peritoneal and pleural cavities, including expression in macrophages and in the omentum. We find that mice lacking CXCL13 have a severe paucity of B cells in the peritoneal and pleural exudates and within the omentum. Using a short-term transfer approach, B1 cell homing to the omentum and peritoneal cavity is found to depend strongly on CXCL13. By parabiosing pairs of adult mice differing in their Ly5 alleles, we demonstrate that B1a and B1b cells circulate to the peritoneum in adult mice. Furthermore, CXCL13-promoted accumulation of cells in the peritoneum appears to be important for promotion of natural antibody production and for local immunity, as CXCL13-deficient mice have markedly

reduced PC-specific natural antibody levels and diminished responses to intraperitoneally injected bacterial antigens.

Results

Decreased body cavity B1 cell numbers in CXCL13$^{-/-}$ mice

Flow cytometric analysis of peritoneal exudate cells (PEC) revealed a dramatic reduction in the frequency of both B1 and B2 cells in CXCL13$^{-/-}$ mice (Figure 1A). As expected, B1a cells predominated in the PEC of control mice, but of the few B1 cells detected in the PEC of CXCL13$^{-/-}$ mice, most were of the CD5$^{lo/-}$ B1b subset (Figure 1B). Enumeration of PEC revealed that CXCL13$^{-/-}$ mice had 50-fold fewer B1a, 8-fold fewer B1b, and 6-fold fewer B2 cells than heterozygous and wildtype controls (Figure 1C), while peritoneal macrophage numbers were unaffected (data not shown). B cell numbers were also markedly decreased in the pleural cavity of CXCL13$^{-/-}$ mice (Figure 1D). Analysis of the lymphocytes present in wildtype omentum revealed a similar population to that of the peritoneal and pleural cavities, including a prominent Mac1^{+} B1 cell population (Figure 1E). B1 cells constituted approximately one third of the omental B cell population, and this tissue also exhibited a strong requirement for CXCL13 in B cell accumulation (Figure 1E). In contrast with these deficiencies, the number of B cells in spleen was not reduced in CXCL13$^{-/-}$ mice (Figure 1F and Table 1). The approach used to identify B1 cells in the body cavities and the omentum was unsuccessful for the spleen due to the large excess of B2 cells and the reduced Mac1 expression of B1 cells in the spleen (30). However, we

were able to enumerate splenic B1a cells by identifying cells with an $IgM^{hi}CD23^{lo/-}B220^{lo}CD5^{+}$ surface phenotype (Figure 1F). Despite their low frequency and in agreement with previous estimates (1), the number of splenic B1a cells was approximately equal to the number detected in the PEC (Table 1). Interestingly, the frequency of B1a cells in the blood of $CXCL13^{-/-}$ mice was increased compared to controls (Table 1), suggesting that CXCL13 may influence B1 cell exit from the bloodstream.

Chemokine responses of peritoneal B cells: B1 cells are highly sensitive to CXCL13

In agreement with previous findings (31-33), B1 cells express CXCR5, with B1a and B1b cells both expressing approximately 2-fold higher amounts than peritoneal B2 cells (Figure 2A). B1a and B1b cells showed robust chemotactic responses to CXCL13 in *in vitro* assays, although the total fraction of responding cells was less than for B2 cells (Figure 2B). B1 cells also responded to CXCL12, but less strongly than to CXCL13, and they showed only very weak chemotactic responses to CCL19 and CCL21 (Figure 2B). Calcium flux analysis showed a similar chemokine response profile, with B1 cells responding strongly to CXCL13 and weakly to CXCL12, CCL19 and CCL21 (Figure 2C). The calcium flux analysis also revealed that all B1 cells were responsive to CXCL13, consistent with the CXCR5 expression profile (Figure 2A and C). Therefore, the finding that fewer B1 cells than B2 cells migrate to CXCL13 may reflect differing abilities of the cells to undergo chemotaxis in the *in vitro* assay rather than a

lower ability of this population of cells to respond. In fact, the sensitivity of B1 cells to CXCL13 is greater than that of B2 cells. Analysis of the dose response curves revealed that B1 cells reached their maximal chemotactic response at lower CXCL13 concentrations than B2 cells (Figure 2B and (33)), and that CXCL13 induces calcium flux in B1 cells at a low concentration that does not induce a response in B2 cells (Figure 2C).

Two sources of CXCL13 within the peritoneal cavity

The deficiency of peritoneal and pleural cavity B cells in $CXCL13^{-/-}$ mice suggested that CXCL13 might function locally in the body cavities. Northern blot analysis revealed CXCL13 expression in total peritoneal and pleural exudate cells (Figure 3A) and in purified peritoneal macrophages (Figure 3B). In contrast to the requirements for CXCL13 expression in spleen (34), CXCL13 expression by peritoneal macrophages was independent of B cells and of LTα, LTβ and TNF (Figure 3A). Comparing CXCL13 levels in different organs of wildtype mice revealed a stronger signal in RNA prepared from PEC than from lymph nodes and spleen (Figure 3A and D). By contrast, CXCL12, CCL19, and CCL21 were expressed at very low to undetectable levels in PEC (Figure 3C).

To characterize the contribution of macrophage-derived CXCL13 to B cell accumulation in the peritoneum, lethally irradiated $CXCL13^{-/-}$ $Ly5^{b}$ mice were reconstituted with wildtype $Ly5^{a}$ bone marrow. Analysis of chimeric animals after 8 weeks of reconstitution showed that more than 95% of the macrophages in the peritoneal cavity were replaced by donor derived $Ly5^{a}$+ cells (data not shown). Analysis of PEC from such bone marrow chimeras revealed significant

restoration of CXCL13 expression (Figure 3D), and showed that hematopoietically derived CXCL13 was sufficient to promote B1 cell accumulation (Figure 3E). Unexpectedly, however, the reconstitution of B1 cells was limited to 30% of the number found in reconstituted wildtype animals (Figure 3E). These findings suggested that CXCL13 was also produced by radiation resistant cells within the peritoneum. Consistent with this, analysis of RNA prepared from the omentum, a peritoneal tissue composed predominantly of mesothelial cells and adipocytes ((18) and see Figure 5A), revealed strong CXCL13 expression (Figure 3F). CXCL13 mRNA was also consistently detectable in the diaphragm, although at much lower levels, whereas little or no signal could be detected in RNA prepared from mesentery or peritoneal wall (Figure 3F and data not shown). Analysis of omentum RNA prepared from bone marrow chimeric mice indicated that a fraction of omental CXCL13 emanates from bone-marrow derived cells, most likely omentum-associated macrophages (Figure 3D). However, when wildtype mice were irradiated and reconstituted with $CXCL13^{-/-}$ bone marrow to eliminate hematopoietic sources of CXCL13, there was a marked loss of CXCL13 expression in the PEC, but little effect on expression by the omentum (Figure 3D). Therefore, a substantial fraction of omental CXCL13 is expressed by radiation resistant cells. Consistent with an important role for CXCL13 produced by radiation resistant cells, B1 cells accumulated in animals reconstituted with $CXCL13^{-/-}$ bone marrow to 75% of the number found in mice reconstituted with wildtype bone marrow (Figure 3E). In summary, these findings establish that both radiation-resistant cells, most likely

non-hematopoietically derived cells, and radiation-sensitive peritoneal macrophages contribute to the production of CXCL13 that controls B1 cell accumulation in the peritoneum.

B1 cells home to the omentum and peritoneal cavity in a CXCL13-dependent manner

Numerous studies have shown that intravenous transfer of fetal liver, bone marrow or peritoneal cells to irradiated recipients allows reconstitution of the peritoneal B1 cell compartment over a period of weeks ((1, 2) and Figure 3F). However, it has not been reported whether mature B1 cells are able to home to the peritoneum. To visualize B1 cell trafficking, we established a short-term adoptive transfer system using CFSE-labeled PEC (Figure 4). By this approach, we found that a notable fraction of transferred B1 cells home to the peritoneal compartment within 24 hours of transfer (Figure 4A,E). Peritoneal B2 cells home to spleen, but were found at a very low frequency in the PEC (Figure 4A and data not shown). In recipients that lack CXCL13, both B1 and B2 cells failed to migrate to the peritoneum, while the spleen contained both populations at frequencies similar to wildtype controls (Figure 4A). The frequency of transferred B1 and B2 cells recovered from the pleural cavity was similar to that of the PEC, and again homing was defective in $CXCL13^{-/-}$ recipients (Figure 4A). Thus, mature B1 cells are selectively recruited to body cavities in a CXCL13-dependent manner.

Transferred B1 cells were also enriched in the omentum, though donor B2 cells were detected at appreciable numbers in this tissue as well (Figure 4A). As observed for the PEC, B cell homing to the omentum was CXCL13-dependent (Figure 4A). To test whether the omentum may function as a portal of B1 cell entry to the peritoneum, we repeated the intravenous transfer of PEC cells and analyzed recipient mice one hour after transfer, a time point when transferred cells are likely to be concentrated at points of compartmental entry. In preliminary studies, we established that the lymphocyte composition and aggregation of cells in the omentum was not affected when peritoneal lavage was performed prior to dissection of the omentum (data not shown), allowing collection of the omentum and PEC from the same animals. Consistent with a role for the omentum in B1 cell recruitment to the peritoneal cavity, transferred B1 cells were readily detected one hour after transfer in the omentum of wildtype, but not $CXCL13^{-/-}$ animals (Figure 4B). The proportion of B1 cells reaching the peritoneal cavity (assessed by recovery in the PEC) at this early time point was minimal, and in some cases undetectable (Figure 4C). Strikingly, while B1 cell homing to the omentum increased little between one and 24 hours (Figure 4D), the proportion of transferred B1 cells found in the PEC increased more than 300-fold (Figure 4E). In longer term transfers, B1 cells continued to gradually accumulate in the PEC in a CXCL13-dependent manner (data not shown). These findings suggest that B1 cells home from the blood stream to the omentum and then pass from the omentum into the peritoneal cavity.

B1 cell homing within the omentum

To examine the pathway of B1 cell homing into the omentum (Figure 5A), we performed whole-mount light and fluorescence microscopy on tissue isolated from recipients of CFSE-labeled PEC. One hour after transfer, $CFSE^+$ cells were found proximal to or within vessels located in omental milky spots (Figure 5B and C). Although we were unable to stain the transferred cells for B cell markers *in situ*, analysis of mechanically dispersed omental cells showed that more than 80% of the $CFSE^+$ cells were B cells (Figure 4 and data not shown). $CFSE^+$ cells were highly enriched in the omentum compared to the adjacent pancreas (Figure 5D). Twenty-four hours after transfer, CFSE-labeled cells were observed to have exited vessels, but remained clustered within milky spots (Figure 5E). In $CXCL13^{-/-}$ recipients, transferred cells were found in decreased numbers within the omentum, mainly associated with vessels scattered throughout the tissue (not shown). Even after 24 hours in $CXCL13^{-/-}$ mice, the cells failed to move out to the periphery of the omentum or to form clusters, instead remaining scattered in a distribution similar to that seen at one hour after transfer (Figure 5F). Fewer pre-existing milky spots were evident in $CXCL13^{-/-}$ omentum, although whether this reflected a lower number of total aggregates or the near absence of lymphocytes is presently unclear. Taken together with the findings above, these data demonstrate that CXCL13 directs B1 cell homing into and within the omentum, and establish the omentum as a port of entry for B1 cell migration to the peritoneal cavity.

Parabiosis reveals B1 cell homing to body cavities in adult mice

The presence of B1 cells in the spleen and blood of wildtype mice, and our finding that B1 cells are elevated in the blood of CXCL13$^{-/-}$ mice (Table 1), and that transferred B1 cells can home from the blood into the body cavities (Figure 4), suggested that B1 cell trafficking normally occurs between blood and peritoneum. To examine the extent of peritoneal B1 cell recirculation in adult animals, pairs of wildtype C57BL/6 (Ly5^{b}) and congenic C57BL/6 Ly5^{a} mice were surgically joined in parabiosis. In previous studies, parabiosed mice have been shown to undergo complete blood exchange approximately ten times per day (35). Analysis eight weeks later revealed the proportion of Ly5^{a}+ B and T cells in lymph nodes was equal in each partner, indicating that these recirculating lymphocytes had reached an equilibrium distribution in the parabiosed mice (Figure 6A), consistent with published results (36). By contrast, more than 90% of the peritoneal macrophages in each partner remained of host origin (Figure 6B), indicating that peritoneal macrophages do not recirculate frequently, and are not rapidly replaced from cells outside of the peritoneum. Compared to the macrophages, peritoneal B1 cells had undergone a significant degree of mixing between partners, although not to the extent observed for peritoneal B2 cells or T cells (Figure 6B). Similar results were obtained for pleural exudate cells (not shown). Importantly, a similar degree of mixing was observed for the B1a and B1b subsets (Figure 6B). As B1a cells are predominantly generated during fetal and neonatal life and are not efficiently generated by adult bone marrow (1, 2), this finding supports the interpretation that B1 cells are able to leave and re-enter

the peritoneal and pleural cavities. While the degree of mixing was not complete, indicating that B1 cells are not freely recirculating, the findings establish that B1 cell homing to the peritoneal cavity occurs in adult animals.

Defective natural antibody production and body cavity immunity in CXCL13$^{-/-}$ mice

To examine the impact of peritoneal and pleural B1 cell deficiency on natural antibody production, we measured levels of PC-binding antibody in serum of unimmunized CXCL13$^{-/-}$ mice. As antibodies containing the T15 idiotype bind PC and are produced exclusively by B1 cells (8), we also measured T15-containing antibodies. Unimmunized CXCL13$^{-/-}$ mice had detectable, but significantly reduced levels of PC-binding antibodies (Figure 7A) and T15-containing antibodies (Figure 7B), despite normal total IgM levels (Figure 7C). CXCL13$^{-/-}$ mice also had normal serum concentrations of IgG and IgA (Figure 7D). These data establish a critical role for CXCL13 in natural antibody production. To further test B1 cell function in the absence of CXCL13, mice were immunized with the R36A streptococcal vaccine. In wildtype C57BL/6 mice, intravenous injection of the R36A vaccine induces a T-independent (TI) antibody response against PC. This response originates in the spleen and is mediated by both T15-expressing B1 cells and a smaller number of marginal zone B cells (30). In contrast, only B1 cells respond to a low dose of intraperitoneally administered vaccine, presumably due to efficient antigen capture within the peritoneum. Consistent with a defect in peritoneal B1 cell function, CXCL13$^{-/-}$

mice had a five-fold reduction in PC-specific antibody, and a ten-fold reduction in T15-containing antibody responses to intraperitoneal injection of the R36A vaccine (Figure 7E). However, $CXCL13^{-/-}$ mice were able to mount a relatively normal response to intravenously injected vaccine, indicating that B1 cells are functional in the spleen of $CXCL13^{-/-}$ mice (Figure 7F and data not shown). Therefore, effective anti-PC responses against intraperitoneally introduced bacterial antigen depends on CXCL13-mediated B1 cell accumulation in the peritoneum.

Discussion

Chemokines have emerged as key regulators of basal lymphocyte trafficking, directing different cell types to microenvironments that maximize their immune surveillance function. Here we have characterized the mechanism of B1 cell homing to body cavities and revealed a critical role for CXCL13 in this process. The pathway we define for homing to the peritoneum involves B1 cells migrating from blood vessels into the omental mesothelial tissue followed by movement into the peritoneal cavity. We propose that B1 cells home to pleural and pericardial cavities by similar pathways. In addition to a homing defect, CXCL13-deficient mice have defective B1 cell function, with reduced natural antibody levels and reduced production of antibody in response to intraperitoneal immunization. Our findings support the conclusions that B1 compartmentalization in body cavities serves to promote natural antibody production and to ensure the

rapid antibody responses needed for protection from low-grade but potentially life-threatening infections of the body cavities.

A surprising finding in this work was the discovery of two independent sources of CXCL13 contributing to B cell accumulation in the peritoneal cavity: macrophages and radioresistant cells. The observation that macrophages can act as a physiologically important source of CXCL13 in the peritoneum raises the possibility that macrophages and related cells, such as DCs, contribute to CXCL13 expression and B cell recruitment at sites of inflammation. Recently it has been found that some types of DC can express CXCL13 (37). Several reports have established that CXCL13 can become upregulated at sites of chronic inflammation (reviewed in (38)) and in one case, the cells expressing CXCL13 were shown to include DCs (33). Reciprocally, our studies strengthen the previous evidence (22, 23) that stromal cells, rather than hematopoietic cells, are the predominant source of CXCL13 in secondary lymphoid tissues as CXCL13 expression was not restored in $CXCL13^{-/-}$ spleen or mesenteric lymph nodes after reconstitution with wildtype bone marrow (see Figure 3D). The nature of the radiation resistant cell type(s) in the peritoneum that produces CXCL13 has been difficult to define as we have so far been unable to detect CXCL13 in peritoneal tissues by immunohistochemistry (not shown). However, the substantial radiation resistant CXCL13 expression in the omentum, as well as weak expression in the diaphragm, supports the conclusion that some type of mesothelial cell produces this chemokine. Consistent with this, mesothelial cells have been found to constitutively express CXCL12/SDF1 (39) and to express

several inflammatory chemokines, including CCL8/IL-8 and CCL2/MCP1, during peritoneal inflammation (40-42).

Early microscopy studies of the omentum of many species, including humans, identified the universal presence of lymphoid aggregates or milky spots (18, 43). Our findings provide strong evidence that B1 cells enter the omentum directly from the blood, entering most efficiently across vessels within the milky spots (see Figure 5). Electron microscopic analysis provided evidence that capillaries present in the lymphoid aggregates are points of cellular traffic (44), and monocytes and neutrophils have been suggested to traverse these vessels (45, 46). The delayed accumulation of transferred B1 cells in the peritoneal exudate compared to their appearance in the omentum (see Figure 4) favors the notion that B1 cells enter the peritoneal cavity from the omentum. During inflammation, macrophages and neutrophils may also follow this route into the peritoneal cavity (46). Microscopy studies have shown that cellular passage between milky spots and the surface of the omentum occurs via fenestrations in the mesothelium overlying the aggregates (18). Taking all the findings together, we conclude that B1 cells use the omentum as a portal of entry to the peritoneal cavity. Milky spots have also been observed in the mediastinal pleura and the pericardium (18, 46, 47). As we found that transferred B1 cells appear in the pleural cavity with similar kinetics to their appearance in the peritoneal cavity, all the findings are consistent with the possibility that mesothelial surfaces of the mediastinal pleura, the pericardium, as well as the omentum, provide passage of B1 cells into the respective body cavities.

Homing to body cavities was strikingly selective for B1, versus B2 cells (see Figure 4). The greater sensitivity of B1 cells to CXCL13 than B2 cells, together with their reduced responsiveness to CCL19 and CCL21 (see Figure 2), may contribute to their selective tropism for body cavities. However, analysis of B cells recruited to a site of ectopic CXCL13 expression did not show evidence for selective recruitment of B1 cells ((48) and unpubl. obs.), indicating that additional mechanisms must contribute to homing specificity. These may include differential selectin- or integrin-mediated adhesion (21). Our experiments do not exclude that CXCL13 contributes to B1 cell accumulation in body cavities by promoting cell retention as well, similar to the role of CXCL12 in precursor cell retention in the bone marrow (28, 49). Interestingly, treatment with neutralizing antibodies to CXCL12 was recently found to reduce B cell numbers in the peritoneum by 2-fold (39). However, this effect was suggested to occur at the level of B1 cell survival rather than homing. It will be interesting to determine whether the few remaining B1 cells that accumulate in the peritoneal cavity of $CXCL13^{-/-}$ mice do so in response to CXCL12.

From studies showing development of B1 cells in *in vitro* fetal liver and bone marrow cultures (50, 51) and, under some conditions, from B2 cells (4, 7), it appears likely that B1 cells acquire many of their phenotypic characteristics outside the body cavities and that they then home to the cavities in a CXCL13-dependent manner. Furthermore, transfers of cells to the peritoneum of lymphocyte deficient recipients have demonstrated that, over long-periods of time, B1 cells are able to leave the peritoneum and appear in the spleen and

some other tissues, in some cases arriving in these sites as antibody secreting cells (11, 52, 53). The ability of B1 cells to localize in the peritoneal cavity of partner mice during parabiosis confirms that B1 cells migrate to the peritoneum in adult animals. Furthermore, the similar mixing of B1a and B1b cells between parabiotic partners, despite the lower production of B1a cells in the adult bone marrow (1), suggests that cells are exiting and re-entering the peritoneum in a pattern of recirculation. A well developed network of lymphatics exists within the peritoneum, including vessels in the omentum and in the diaphragm (18, 54), providing a possible exit route for B1 cells. The purpose of B1 cell circulation is not yet clear, but the recent demonstration that B1 cells work together with marginal zone cells in T-independent responses to systemic bacteria (30) suggests that recirculation allows the unique B1 repertoire to be available for responses against infections not involving the body cavities.

B1 cells are well characterized as a source of natural antibodies that are important for defense against infections by bacteria (13, 14, 17) and viruses (15, 16). PC-specific natural antibody levels were substantially reduced in CXCL13$^{-/-}$ mice, despite normal B1 cell numbers and function in the spleen (Table 1 and Figure 7E). Thus, B1 cell homing to body cavities appears to be critical for their role in innate immunity, and factors peculiar to the body cavity microenvironments are presumably necessary for stimulating natural antibody production. These factors may be represented by a specialized local cytokine milieu (2, 39, 55, 56), and low level exposure to commensal bacterial flora. Consistent with the latter possibility, germ-free BALB/c mice have markedly

reduced levels of serum anti-PC antibodies compared to conventionally reared animals (57, 58). Antibodies against human blood group antigens and related carbohydrates found in lower mammals, but not humans, are responsible for acute responses against mismatched transfusions and xenotransplanted organs. These clinically important natural antibodies are also believed to be derived from peritoneal B1 cells (59) that are stimulated by commensal bacteria (60). Peritoneal localization may also function to sequester B1 cells away from circulating autoantigens that might otherwise lead to their deletion (5, 61). Although our studies support the view that B1 cells recirculate via the bloodstream, this occurs at a lower rate than for B2 cells, and it seems possible that only a fraction of B1 cells undergo recirculation. This may therefore permit B cells that recognize circulating autoantigens to persist in the peritoneal compartment for longer periods than is the case for cells recirculating between secondary lymphoid organs. Strategies to inhibit localization of B cells within body cavities may therefore be clinically relevant in both transplantation and autoimmunity.

Finally, the unique compartmentalization of B1 cells places them in a position to intercept infectious agents that enter the body cavities. Intraperitoneally or intrapleurally injected antigens are rapidly captured within the omentum or mediastinal pleura, respectively, and these tissues likely contribute to the efficiency of body cavity immunity by concentrating antigens in sites of B1 cell traffic. Intraperitoneally injected streptococcal vaccine may mimic intestinal injury or leakage that would result in the release of bacteria into the peritoneal

cavity, and the defective antibody response of CXCL13$^{-/-}$ mice demonstrates the importance of B1 cell homing for effective body cavity immunity. It is interesting to speculate that the earliest function of CXCL13 may have been to recruit primitive B lymphocytes to body cavities for T-independent responses, prior to its involvement in the more complexly organized secondary lymphoid tissues that support T-dependent antibody responses. Further characterization of mice that are selectively deficient in body cavity B cells should lead to a better definition of mechanisms of natural antibody production and should improve our understanding of the importance of mounting local antibody responses for protection of these cavities.

Experimental Procedures

Mice and Parabiosis

Wildtype, RAG-1-/-, TNF-/-, and LTβ-/- mice were from a C57BL/6 (B6) colony. Additional wildtype B6, B6-CD45.1 (Ly5a+), and LTα-/- mice on a mixed B6×129 background were purchased from Jackson Laboratories (Bar Harbor, ME). CXCL13-/- mice were generated as described (24) and maintained on a B6×129 strain background, or backcrossed ten times to the B6 background. Compared to B6×129 mice, wildtype B6 mice have increased numbers of PEC B2 cells, but no strain differences were observed for the CXCL13-dependence of B1 and B2 cell homing, accumulation in body cavities, or antibody production. IgHb/b mice were used for all serum antibody measurements. Bone marrow chimeric mice were generated as described (29). Recipient mice were twice

irradiated (550 rad doses separated by 3 hours) using a cesium source, and 2-5×10^6 bone marrow cells were subsequently injected into each recipient via the lateral tail vein. Mice were given antibiotics (1.1 g/L neomycin, 110mg/L polymixin B sulfate) in the drinking water until sacrifice after 8-16 weeks of reconstitution. The donor origin of lymphocytes in the chimeric mice was confirmed using mismatched allelic markers (Ly5a/b or Ly9.1). Consistent with previous reports, bone marrow transplantation reconstituted peritoneal B1b cells, but few B1a cells (1). CXCL13$^{-/-}$ fetal liver cells reconstituted B1a and B1b cells equally in wildtype recipients. All mice were maintained in specific pathogen-free conditions at the University of California, San Francisco, except parabiotic mice which were housed at the University of Georgia (Athens, GA). Six-week old male B6 and B6-Ly5^a mice were matched for body weight and surgically joined by parabiosis as described (35). Blood exchange was confirmed for all pairs two weeks post-surgery by injection of Evan's blue dye as described (35).

Cell Preparation and Flow Cytometry

Peritoneal exudate cells were isolated by flushing the peritoneal cavity with 4-6mL of PBS supplemented with 0.5% w/v bovine serum albumin (BSA). For pleural exudate cells, 1-2mL of PBS/BSA were injected through the diaphragm. Cell suspensions were prepared from mouse omentum, spleen, and lymph nodes by gentle mechanical disruption and passed through a 70μm nylon mesh. Care was taken to avoid spleen contamination in omental preparations and vice versa. Blood was collected from the inferior vena cava (i.v.c.) to avoid

contamination with body cavity lymphocytes, and red blood cells were lysed with Tris/ammonium chloride. Cells were resuspended in media (RPMI, 10mM HEPES, 50 IU/mL penicillin, 50µg/mL streptomycin) with 2% fetal calf serum (FCS) and counted using a haemocytometer (PEC, pleural EC, omentum) or Coulter counter (spleen, lymph node, blood), and surface stained for flow cytometry using the following antibodies: anti-B220-FITC, anti-B220-PE, anti-B220-Cy5PE, anti-Mac1-biotin, anti-Mac1-PE, anti-F4/80-PE, anti-CD4-FITC (Caltag Laboratories, Burlingame, CA); goat anti-mouse IgM-FITC (Jackson Immunoresearch Inc, West Grove, PA); anti-CD5-biotin, anti-CD23-biotin, anti-IgD-FITC, anti-$Ly5^a$-PE, anti-$Ly5^a$-FITC (A20), anti-B220-APC, anti-B220-PerCP, and anti-CD8-PerCP, goat anti-rabbit IgG-biotin, streptavidin (SA)-APC, and SA-Cy5PE (BD Pharmingen, San Diego, CA), and affinity-purified rabbit anti-mouse CXCR5. Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Files were acquired using CELLQuest software (Becton Dickinson) and analyzed using FlowJo software (TreeStar Inc., San Carlos, CA). For sorting of peritoneal macrophages and B cells, pooled PEC from six B6 mice were stained with anti-IgM-FITC, anti-IgD-FITC, anti-F4/80-PE, and anti-Mac1-biotin/streptavidin-APC, and $Mac1^+F4/80^+IgM/D^-$ macrophages and $IgM/D^+F4/80^-$ B cells from were sorted to >90% purity using a MoFlo fluorescence-activated cell sorter (Cytomation, Fort Collins, CO).

Chemotaxis and Calcium Flux Assays

PEC were prepared in media/0.5% BSA. For chemotaxis assays, chemokines were diluted in media/BSA and aliquoted at 580µL/well into 24-well tissue culture plates. 5µm pore transwell inserts (Corning Costar Corp., Acton, MA) containing 10^6 PEC in 100µL media/BSA were suspended in the wells and incubated for 3 hours at 37°C, 5% CO_2. In some assays, PEC were preincubated at 37°C for 1 hour at 10^7 cells/mL in media/2% FCS to allow chemokine receptor resensitization. Spleen cells from $Ly5^a$+ or Ig-transgenic mice were mixed with PEC in some assays to provide an internal control for normal B2 cell migration. Spleen and PEC B2 migration was very similar in each case. Cells transmigrating to the lower chamber were enumerated by collecting events for a fixed time (60s) on the FACSCalibur. The percentage of input cells that transmigrated was calculated by counting the contents of wells containing 2×10^5 directly aliquoted input cells in the same way. To determine what subsets of cells transmigrated, a fraction of the cells in the lower chamber were stained with anti-CD5-biotin/SA-APC, anti-B220-TC, and anti-Mac1-PE (and in some cases anti-$Ly5^a$-FITC or anti-IgD^a-FITC) and analyzed on the FACSCalibur. To detect calcium signaling, PEC were loaded with 1µg/mL fluo-3 (Molecular Probes, Eugene, OR) during 30 minutes incubation at 37°C in media/5% FCS. After the first 20 minutes, anti-B220-APC and anti-Mac1-PE were added. Cells were washed twice and resuspended in media/5% FCS at ~5×10^6 cells/mL. For each chemokine response, an aliquot of labeled cells was analyzed on a FACSCalibur flow cytometer for 20 seconds, mixed with chemokine, and immediately returned to

the flow cytometer for an additional 2-3 minutes. Chemokine concentration was controlled by adding 120 µL of cell suspension to 30µL of chemokine diluted to five times the desired concentration in PBS. PBS alone did not induce any change in fluo-3 fluorescence. CXCL13 and CCL19 (R&D Systems, Minneapolis, MN), and CXCL12 (Peprotech, Rocky Hill, NJ) were purchased. HIS_6-tagged CCL21 was produced and purified as described (29).

Northern Blot Analysis

To produce intact RNA from the omentum, the omental fat pads and mesothelial sheet were carefully dissected, discarding all pancreatic tissue. PEC, pleural EC, and sorted PEC populations were pelleted by centrifugation (6 minutes, 300g). Total RNA was prepared from cell pellets and homogenized tissues using Trizol Reagent (Life Technologies Inc., Grand Island, NY) according to the manufacturer's instructions. RNA was re-precipitated with ethanol/0.5M LiCl to remove contaminating DNA. 10µg of RNase-free glycogen (Life Technologies) was used as carrier in precipitations for all PEC samples and others with low expected RNA yield. For Northern blot analysis, 5-10µg of RNA was subjected to gel electrophoresis, transferred to Hybond N+ membranes (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), and probed using randomly-primed ^{32}P-labeled mouse cDNA probes as described (34). A *cxcl13* exon 2-specific probe was generated by PCR amplification of mouse genomic DNA using the primers: 5'-CGTCTATGTTCTTTGTCCAATGGG-3' and 5'-CTTACACAACTTCAGTTTTGGGGC-3'. To control for loading and RNA

integrity, membranes were reprobed with a mouse elongation factor 1α (EF-1α) probe.

Adoptive transfers and whole-mount microscopy

Donor PEC were fluorescently labeled by incubating cells for 10-15 minutes at 37°C in media/2%FCS and 0.2μM (for flow cytometry studies) or 10μM (for microscopy studies) carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) and washed with media/2%FCS. Donor cells were analyzed by flow cytometry to calculate the percentage of B1 cells, and 0.5 to 3×10^7 total PEC were injected into each recipient via the lateral tail vein. For whole-mount microscopy, omenta were dissected from recipient mice, placed on a slide, and covered with PBS. In some experiments, the omentum was soaked for 5-10 minutes in hematoxylin to stain nuclei and washed with PBS for viewing. Omenta were viewed with a fluorescence microscope (DMRL; Leica, Wetzlar, Germany) and digital images were acquired with a video camera (Optronics MDEI850 CCD, Optronics Engineering, Goleta, CA) and processed with Photoshop software (Adobe Systems, Mountain View, CA).

Immunizations and ELISAs

For responses against streptococcal antigen, 12-15 week-old male mice were immunized intravenously (tail vein) or intraperitoneally with 10^7 heat-killed pepsin-treated *S. pneumoniae* strain R36A ((30), gift of John Kearney, University of Alabama, Birmingham), and serum was prepared from blood collected from

the tail vein five days later. For natural antibody measurement, serum was prepared from unimmunized mice. For measurement of PC-specific and T15-containing antibody detection, microtiter plates were coated overnight at 4°C with PC-BSA (5μg/mL; Biosearch Technologies Inc., Novato, CA) or the anti-T15 idiotype monoclonal antibody AB1-2 (1μg/mL; gift of J. Kearney) diluted in carbonate buffer (pH 9.6). Plates were washed with PBST and blocked for 1-2 hours with 5% BSA in PBST at room temperature. Serum, controls, and detection antibodies were diluted in PBST/1% BSA. Serum dilutions were incubated in the coated wells for 1-2 hours at room temperature, followed by washing with PBST. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL), and development with the HRP substrate, ABTS (Southern). The PC-specific T15-containing monoclonal IgM antibody, BH8 (a gift of J. Kearney), was used as a standard for quantitation. For unimmunized mice, high concentrations of serum (1:5, 1:25, 1:125, 1:625 dilution) were used. RAG-$1^{-/-}$ serum diluted 1:5 produced no signal above buffer-only controls. For detection of total serum immunoglobulins, plates were coated with goat anti-mouse Ig(M+G+A) (10μg/mL; Southern) and bound antibodies were detected with HRP-conjugated purified antisera for IgM, IgG1, IgG2b, IgG3, or IgA (Mouse Immunoglobulin Clonotyping Kit; Southern). Purified mouse immunoglobulins (Southern) were used as standards for quantitation. Statistical analyses were done with Statview software (v5.0; SAS Institute Inc., Cary, NC).

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Table 1

Altered B1a cell distribution in CXCL13-deficient mice.

Four color flow cytometric analysis was used to identify $IgM^{hi}CD23^{lo/-}B220^{lo}CD5^{+}$ B1a and $IgM^{+}CD23^{+}B220^{+}CD5^{-}$ B2 cells in PEC, spleen, and blood of $CXCL13^{+/-}$ mice and their $CXCL13^{-/-}$ littermates (see also Figure 1F). Numbers represent mean ± standard deviation for absolute cell numbers from eight mice each (PEC and spleen) or percentage of total cells from seven mice each (blood). *, different than controls ($p<0.005$; unpaired student's t-test).

Genotype	PEC B1a ($\times10^{-4}$)	Spleen B1a ($\times10^{-4}$)	Spleen B2 ($\times10^{-6}$)	Blood B1a (%)
$CXCL13^{+/-}$	19±7	31±16	38±11	0.11±0.05
$CXCL13^{-/-}$	0.2±0.2*	36±20	43±15	0.40±0.17*

Figure 1

Reduced B cell numbers in the peritoneal and pleural cavities and the omentum of CXCL13$^{-/-}$ mice.

(A) Flow cytometric analysis of Mac-1 and B220 expression by size-gated PEC. Boxes demarcate Mac-1^{+}B220^{lo} B1 and Mac-1^{-}B220^{hi} B2 subsets, and numbers represent their mean percentage of total PEC cells from twenty CXCL13$^{+/\pm}$ (wildtype or heterozygous) mice (left) and twenty CXCL13$^{-/-}$ littermates (right). *(B)* CD5 expression on peritoneal B1 cells from CXCL13$^{+/-}$ (blue) and CXCL13$^{-/-}$ (red) mice. Brackets demarcate the CD5^{+} B1a and CD5^{-} B1b subsets. *(C-E)* Mean number of B cell subsets in PEC *(C)*, pleural exudate cells *(D)*, and omentum *(E)* of CXCL13$^{+/\pm}$ (blue bars) and CXCL13$^{-/-}$ (red bars) mice. Error bars indicate 95% confidence intervals ($n \geq 24$ for PEC, $n \geq 7$ for pleural EC, $n \geq 8$ for omentum). Differences in numbers of B cells between CXCL13$^{+/\pm}$ and CXCL13$^{-/-}$ mice are statistically significant for all subsets in all tissues ($p<0.005$). No differences in PEC B cell numbers were detected between wildtype and heterozygous mice.

(F) Four color flow cytometric analysis to detect B1a cells in the spleen. Analysis of IgM and CD23 expression by size-gated splenocytes (left panel). IgM^{hi}CD23$^{lo/-}$ cells demarcated by the box labeled G1 (left panel) were analyzed for expression of B220 and CD5 (center and right panels). B220^{lo}CD5^{+} B1a cells (demarcated by boxes) are detected in both CXCL13$^{+/-}$ (center) and CXCL13$^{-/-}$ (right) mice. This gating strategy was used for enumeration of B1a cells in Table 1.

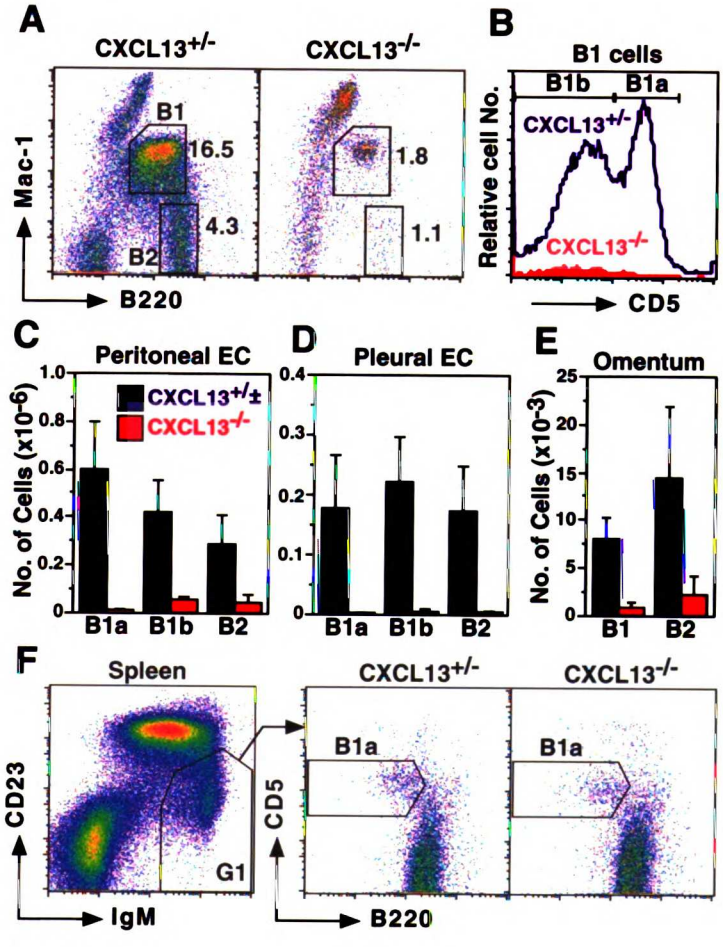

A
CXCL13+/-
CXCL13-/-
B1
16.5
4.3
B2
1.8
1.1
Mac-1
B220
B
B1 cells
B1b
B1a
CXCL13+/-
CXCL13-/-
Relative cell No.
CD5
C
Peritoneal EC
CXCL13+/±
CXCL13-/-
No. of Cells (x10-6)
B1a B1b B2
D
Pleural EC
B1a B1b B2
E
Omentum
No. of Cells (x10-3)
B1 B2
F
Spleen
CXCL13+/-
CXCL13-/-
B1a
B1a
CD23
G1
IgM
CD5
B220

Figure 2

Heightened CXCR5 expression and CXCL13 sensitivity of B1 cells.

(A) CXCR5 expression on PEC B1a (bold line), B1b (solid line), and B2 (dashed line) cells. B1 cells stained with non-specific rabbit IgG is shown as a control (ctl, dotted line).

(B) Chemotaxis of PEC B1 cells (solid line, hatched bars) and B2 cells (dashed line, open bars) in response to the indicated chemokines (CXCL12, 0.3μg/mL; CCL19, 0.2μg/mL; CCL21, 0.8μg/mL). Data are representative of four experiments.

(C) Calcium flux of fluo-3-loaded $B220^{lo}Mac1^{+}$ B1 cells (left) and $B220^{hi}Mac1^{-}$ B2 cells (right) in response to the indicated chemokines added 20 seconds after the start of data collection. Chemokine concentrations are shown in parentheses. Splenic B2 cells responded equally to PEC B2 cells in chemotaxis and calcium flux assays (not shown).

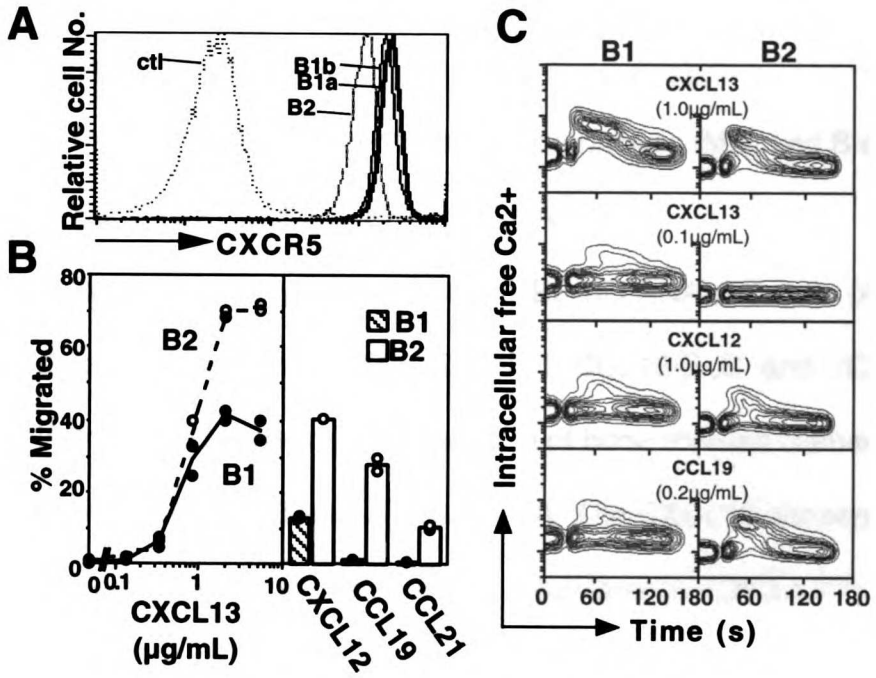

A
Relative cell No.
ctl
B1b
B1a
B2
CXCR5
B
% Migrated
80
60
40
20
0
B2
B1
0 0.1 1 10
CXCL13
(μg/mL)
B1
B2
CXCL12
CCL19
CCL21
C
B1
B2
CXCL13
(1.0μg/mL)
CXCL13
(0.1μg/mL)
CXCL12
(1.0μg/mL)
CCL19
(0.2μg/mL)
Intracellular free Ca2+
0 60 120 180 60 120 180
Time (s)

Figure 3

Two sources of CXCL13 contribute to peritoneal B1 cell accumulation.

(A) Northern blot analysis of total RNA from lymph node (LN) and peritoneal exudate cells (PEC) of wildtype mice and the indicated mutants probed to detect CXCL13 mRNA. Elongation-factor-1α (EF-1α) hybridization indicates amount of RNA in each lane. WT, wildtype; LT, lymphotoxin; TNF, tumor necrosis factor; RAG, recombinase activating gene.

(B) Total RNA from FACS-sorted peritoneal macrophages (Mø) and B cells (B) was probed to detect CXCL13 and EF-1α mRNA.

(C) Northern blot of wildtype lymph node and PEC (P) RNA shown in *(A)*, reprobed to detect expression of CXCL12/SDF-1, CCL19/ELC, and CCL21/SLC.

(D) Total RNA from PEC, spleen, and omentum of bone marrow chimeric mice was probed to detect CXCL13 and EF-1α mRNA. The CXCL13 genotype of donor and recipient mice are indicated above each lane (+, $CXCL13^{+/\pm}$; -, $CXCL13^{-/-}$).

(E) Mean number of B1 cells in PEC of bone marrow chimeras counted and analyzed as in Figure 1. Genotypes of donor and recipient mice are indicated as in *(D)*. Error bars, 95% confidence intervals. The number (n) of each type of chimera analyzed is indicated.

(F) Total RNA from the indicated tissues or cells of a wildtype mouse were probed to detect CXCL13 and EF-1α mRNA. A weak band was consistently obtained for CXCL13 mRNA in diaphragm tissue of several mice.

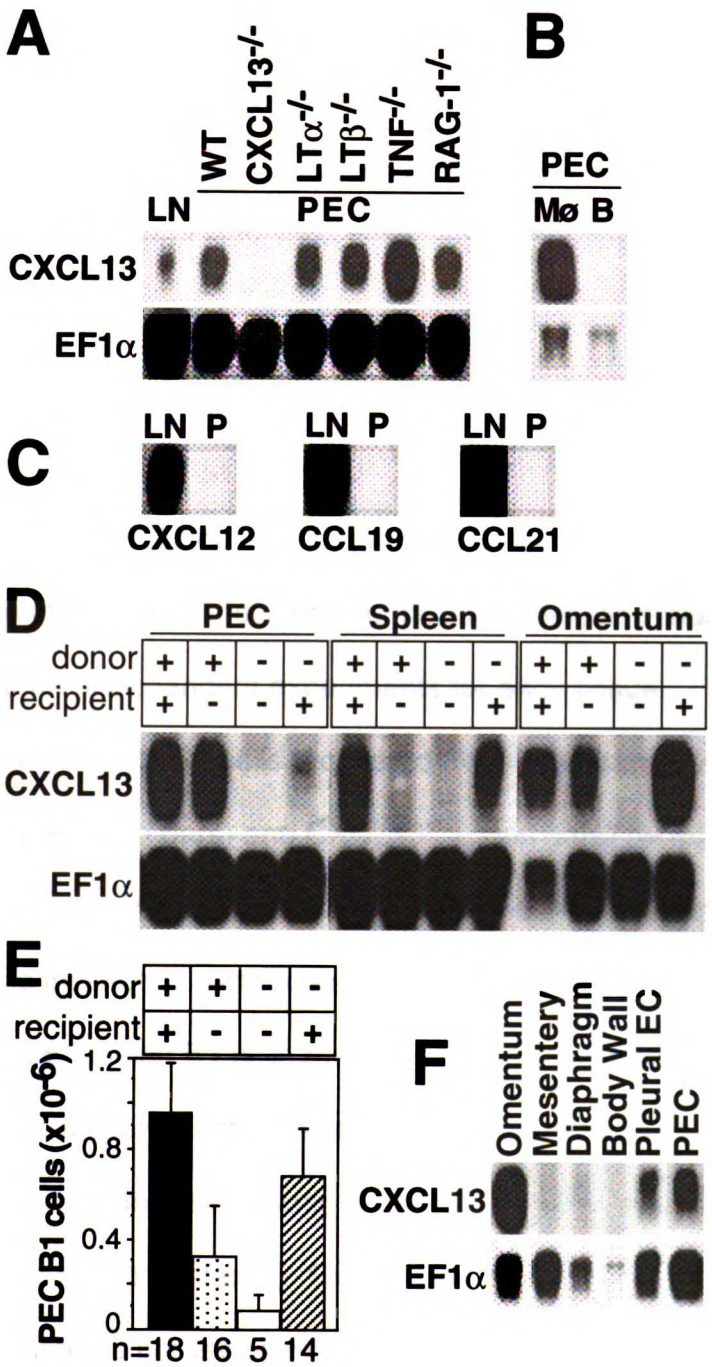

A
WT
CXCL13-/-
LTα-/-
LTβ-/-
TNF-/-
RAG-1-/-
LN
PEC
CXCL13
EF1α
B
PEC
Mø B
C
LN P
LN P
LN P
CXCL12
CCL19
CCL21
D
PEC
Spleen
Omentum
donor
recipient
CXCL13
EF1α
E
donor
recipient
PEC B1 cells (x10-6)
1.2
0.8
0.4
0
n=18 16 5 14
F
Omentum
Mesentery
Diaphragm
Body Wall
Pleural EC
PEC
CXCL13
EF1α

Figure 4

CXCL13-dependent selective recruitment of adoptively transferred B1 cells to the peritoneal and pleural cavities and the omentum.

(A) Representative flow cytometric analyses of CFSE fluorescence and Mac-1 expression of IgM^+ size-gated cells from PEC, spleen, pleural exudate cells, and omentum of wildtype (WT, upper panels) and $CXCL13^{-/-}$ (KO, lower panels) recipients of CFSE-labeled wildtype PEC, twenty-four hours after transfer. Boxes demarcate the positions of large Mac-1^+IgM^{hi} donor-derived B1 cells and smaller Mac-1^-IgM^+ donor-derived B2 cells, and numbers in each panel indicate the mean percentage of total cells that were donor B1 (upper) or donor B2 (lower) cells for at least three independent experiments for each tissue.

(B-E) Enumeration of donor B1 cells recovered from omentum *(B and D)* or PEC *(C and E)* of wildtype (filled bars) and $CXCL13^{-/-}$ (open bars) recipients one hour *(B and C)* or ~24 hours *(D and E)* after transfer. To normalize differences in the number of B1 cells injected in each transfer experiment, data are expressed as the number of CFSE-labeled B1 cells recovered in each recipient per 10^5 B1 cells intravenously transferred into that recipient. Bars and error bars represent the mean and 95% confidence intervals, respectively. Note the increased scale for PEC at 24 hours after transfer *(E)*. Data are representative of at least 3 independent experiments per recipient tissue.

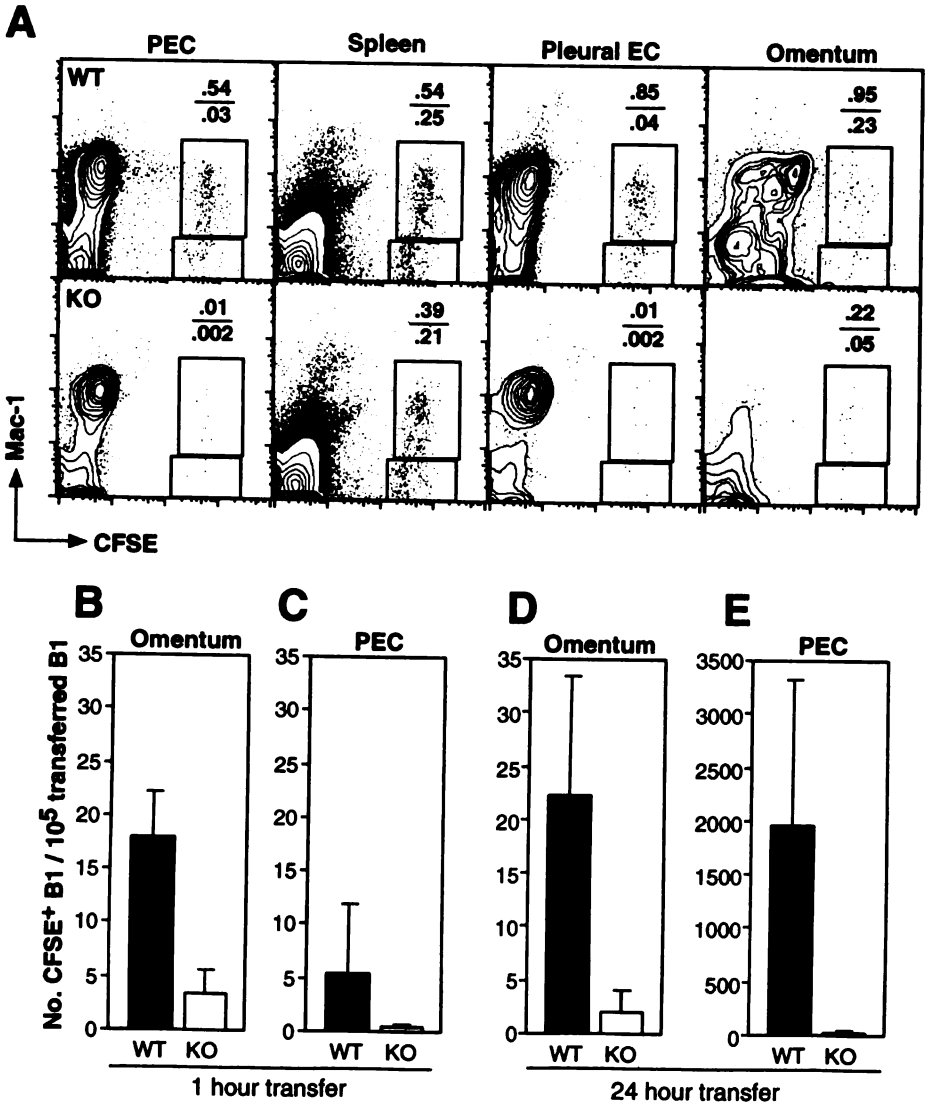

A
PEC
Spleen
Pleural EC
Omentum
WT
KO
.54
.03
.54
.25
.85
.04
.95
.23
.01
.002
.39
.21
.01
.002
.22
.05
Mac-1
CFSE
B
C
D
E
Omentum
PEC
Omentum
PEC
No. CFSE+ B1 / 10^5 transferred B1
WT KO
1 hour transfer
24 hour transfer

Figure 5

B cell homing within the omentum.

(A) A photograph of the mouse omentum (Om) in situ four hours after intraperitoneal injection of carbon particles (India ink). The efficient uptake of particulate antigens by the omentum is illustrated by the concentration of black staining in the omental fat pads. The transparent mesothelial sheet of the omentum connects these fat pads to the spleen (Sp), stomach (St), pancreas (P), and colon (C).

(B-E) Whole-mount microscopic images of the omentum of recipients of CFSE-labeled (green fluorescent) peritoneal exudate cells. *(B)* Light transmission and *(C)* fluorescent images of an omental fat pad (fp) and a small portion of the mesothelial sheet (mes) of a wildtype recipient one hour after transfer. Arrows indicate transferred cells in vessels associated with milky spots (ms). Arrowheads indicate infrequent cells in large vessels of the omentum. Arrows and arrowheads are located in identical positions in C and D for alignment of the images. *(D)* Higher magnification of a milky spot with vessel-associated transferred cells one hour after transfer. The omentum was stained with hematoxylin to highlight nuclei (black). *(E)* The omentum of a wildtype recipient 24 hours after cell transfer. *(F)* The omentum of a CXCL13$^{-/-}$ recipient 24 hours after cell transfer. Data are representative of at least three recipients at each time point.

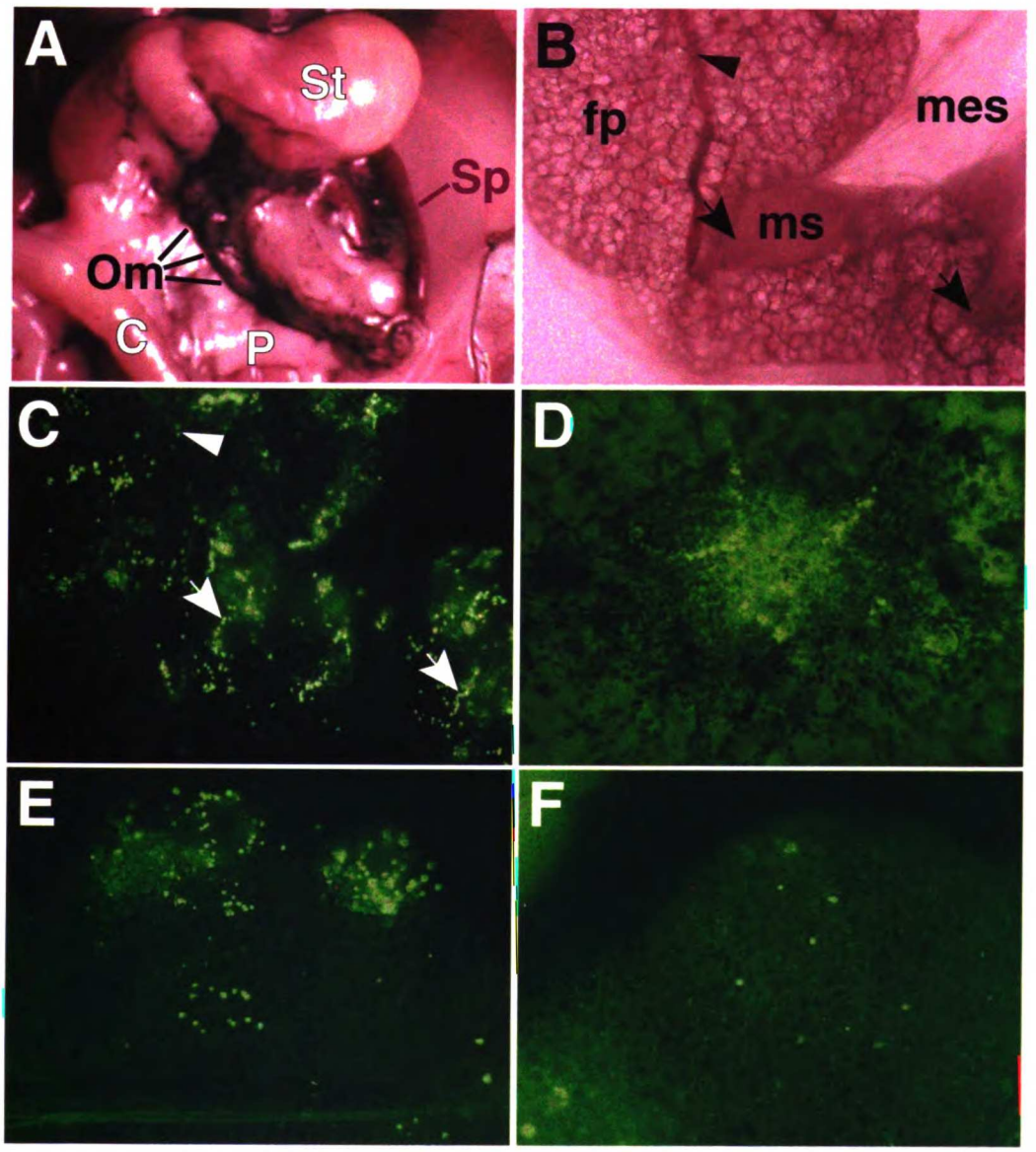

A
St
Sp
Om
C
P
B
fp
mes
ms
C
D
E
F

Figure 6

Mixing of B1 cells between parabiotic mice with shared blood circulation.

Five parabiotic pairs, each consisting of one C57BL/6 $Ly5^b$-expressing mouse and one congenic C57BL/6 $Ly5^a$-expressing mouse, were analyzed 8 weeks after surgical anastomosis. For each cell type, the mean percentage of $Ly5^{a+}$ cells in the $Ly5^b$ partner (open bars) and in the $Ly5^a$ partner (filled bars) is shown. Error bars represent 95% confidence intervals. Data are representative of two independent experiments.

(A) Equilibrium distribution of $Ly5^{a+}$ lymph node (LN) B2 and T cells in parabiotic mice.

(B) Distribution of $Ly5^{a+}$ macrophages (Mø), B2, T, B1a, and B1b cells in peritoneal exudate cells (PEC) of parabiotic mice.

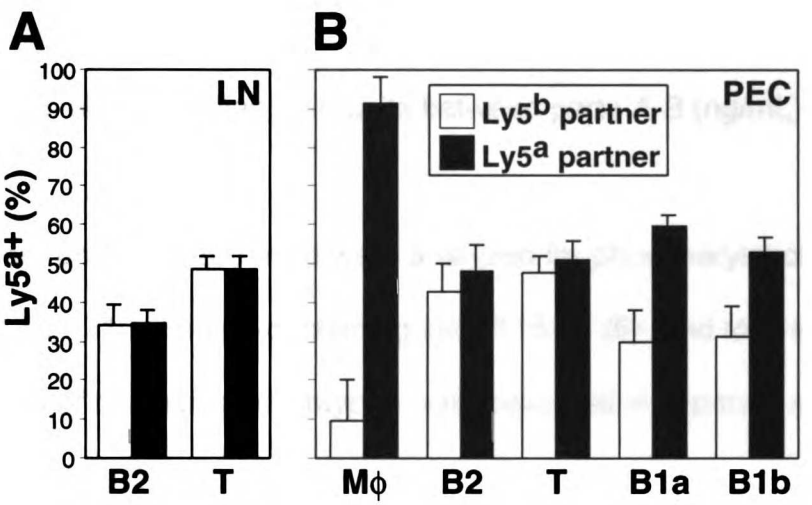

A
B
LN
PEC
Ly5a+ (%)
100
90
80
70
60
50
40
30
20
10
0
B2
T
Ly5b partner
Ly5a partner
Mϕ
B2
T
B1a
B1b

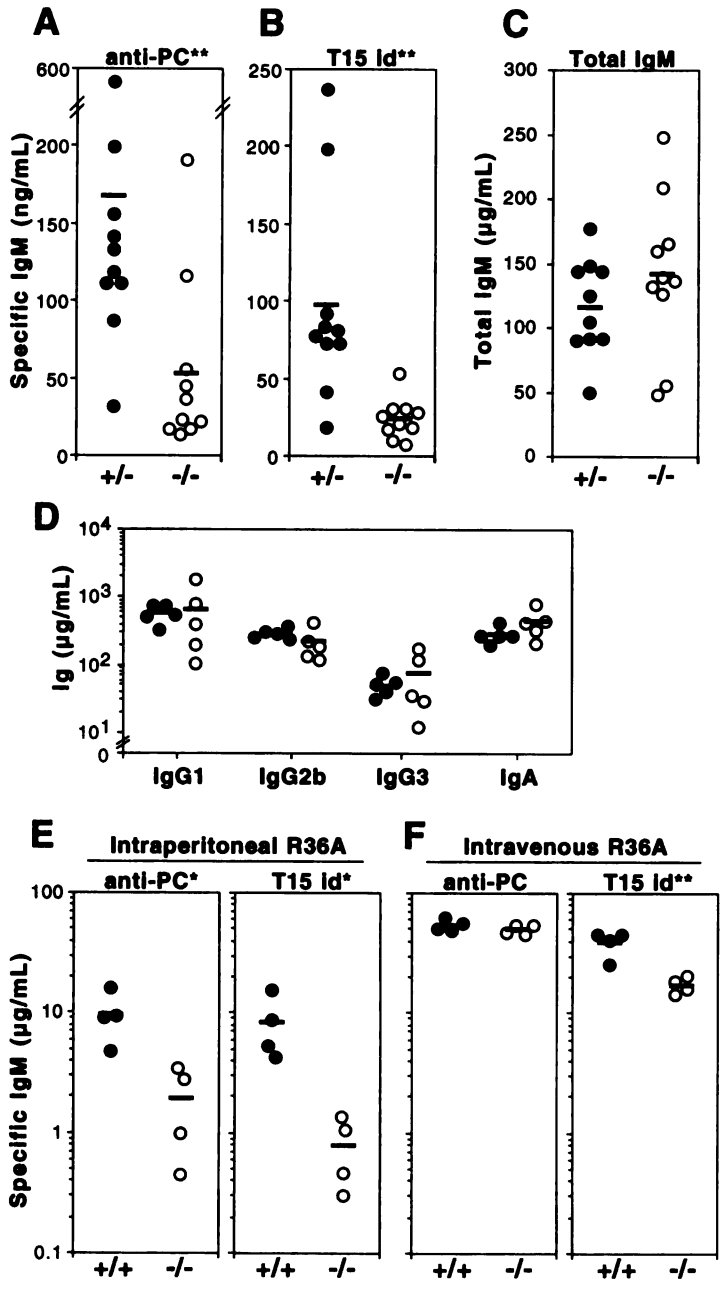
Figure 7

Defective natural antibody production and body cavity immunity in CXCL13$^{-/-}$ mice.

ELISA measurements of serum immunoglobulin (Ig) levels in CXCL13$^{+/\pm}$ (filled circles) and CXCL13$^{-/-}$ (open circles) mice. Lines indicate the mean value for each data set. Statistically significant differences between CXCL13$^{+/\pm}$ and CXCL13$^{-/-}$ antibody levels are indicated by asterisks (*, $p<0.05$; **, $p<0.005$; student's T-test). Note the change in units between parts *A-B* (ng/mL) and parts *C-F* (µg/mL).

(A-D) Sera from unimmunized mice were analyzed for phosphorylcholine-specific IgM (anti-PC) *(A)*, T15 idiotype-containing IgM (T15 id) *(B)*, and total IgM *(C)* in parallel. Total Ig of the indicated isotypes was measured in separate experiments *(D)*.

(E-F) Sera from mice immunized five days previously by intraperitoneal *(E)* or intravenous *(F)* injection of R36A streptococcal vaccine were analyzed for PC-specific IgM (left panels) and T15-containing IgM (right panels).


A
anti-PC**
Specific IgM (ng/mL)
600
200
150
100
50
0
+/-
-/-
B
T15 Id**
250
200
150
100
50
0
+/-
-/-
C
Total IgM
Total IgM (µg/mL)
300
250
200
150
100
50
0
+/-
-/-
D
Ig (µg/mL)
10^4
10^3
10^2
10^1
0
IgG1
IgG2b
IgG3
IgA
E
Intraperitoneal R36A
anti-PC*
T15 Id*
Specific IgM (µg/mL)
100
10
1
0.1
+/+
-/-
+/+
-/-
F
Intravenous R36A
anti-PC
T15 Id**
+/+
-/-
+/+
-/-

Chapter 5

Conclusions and Discussion

These studies establish an essential role for the chemokine CXCL13 in the control of lymphocyte homing to environments that promote their immune function. Acting through its receptor, CXCR5, CXCL13 directs B cell homing to follicles in all secondary lymphoid organs. In addition, I found that CXCL13 is critical for follicle homeostasis. CXCL13 induces LTα1β2 expression on B cells, generating a chemokine-cytokine positive feedback loop between the recruitment of B lymphocytes and the maintenance of the follicular stroma that produce CXCL13. In germinal centers, CXCL13-independent signals are sufficient to induce high levels of LTα1β2 expression and the development of FDC networks. Lymph node and Peyer's patch organogenesis is also directed by CXCL13, suggesting that the mechanisms that control secondary lymphoid organ homeostasis may also operate in their development. In addition to the dominant role of CXCL13 in B cell homing, I provide evidence that the acquisition of responsiveness to CXCL13 and decreased responses to T zone chemokines control T cell migration into follicles during T cell-dependent antibody responses. Finally, I characterize the homing of B1 cells, demonstrating that even these specialized B cells that reside principally outside of secondary lymphoid organs depend on CXCL13 for their homing and immune function.

The follicles of secondary lymphoid organs are sites of continuous B cell traffic as naïve B cells recirculate throughout the body, surveying for a cognate antigen for their immunoglobulin specificity. This immune surveillance mechanism is facilitated by FDCs that efficiently capture antigenic immune complexes, concentrating them within follicles. Here, I have demonstrated that a chemokine produced by FDC and other follicular stromal cells, CXCL13, is essential for directing B cell migration into follicles (Chapter 2). It was previously reported that the CXCL13 receptor, CXCR5, is required for B cell homing to follicles in spleen and Peyer's patches, but not lymph nodes (1). However, my experiments using both CXCL13$^{-/-}$ mice and CXCR5$^{-/-}$ mice established that homing to lymph node follicles is also dependent on CXCL13 and CXCR5. In adoptive transfers, CXCR5$^{-/-}$ B cells do colocalize with wildtype B cells in the splenic marginal zone and in medullary cords and areas adjacent to follicles in lymph nodes, but they are not able to enter FDC-containing follicles (Chapter 2).

B cells do not organize into polarized follicles in CXCL13$^{-/-}$ mice, instead forming a ring at the periphery of lymph nodes and extending into the marginal zone in splenic white pulp cords. A closer analysis of these segregated B cell areas revealed that primary follicle FDC are absent in CXCL13$^{-/-}$ mice. Previous reports identified B cells as an important source of LTα1β2 that is required for the homeostasis of the FDC network, but LTα1β2 expression had only been seen on activated B cells (2). Vu Ngo established that naïve B cells express detectable levels of LTα1β2 on their surface, and that LTα1β2 levels are increased in secondary lymphoid organs compared to the blood. Using the CXCL13$^{-/-}$ mice

that I generated, this expression was shown to be CXCL13-dependent (Chapter 2). CXCL13 can induce LTα1β2 expression on primary B cells *in vitro*, and previous studies established that LTα1β2, in turn, is required for CXCL13 expression in follicles (3). Taken together, these data establish a positive feedback loop that controls lymphoid follicle homeostasis. However, a low level of lymphotoxin is maintained on B cells of CXCL13$^{-/-}$ mice, and the disruption of splenic architecture is more severe in lymphotoxin-deficient mice compared to the CXCL13$^{-/-}$ mice. Furthermore, TNF is also required for follicular organization of B cells, and this cytokine is also delivered by B cells (4). Further studies are needed to clarify the regulation of both lymphotoxin and TNF in secondary lymphoid organs.

An increased number of B cells were found in the T zones of CXCL13$^{-/-}$ spleen and lymph nodes. However, considering the absence of CXCL13 and the ability of B cells to chemotax in response to CCL19 and CCL21, the degree of B cell entry into T zones in CXCL13$^{-/-}$ mice is surprisingly small. The access of lymphocytes to T zones and follicles may be strongly affected by small differences in their chemokine responsiveness, especially when these cells must compete with other lymphocytes with a higher degree of responsiveness or greater chemokine sensitivity. For example, the stronger CCL19 and CCL21 responsiveness of T cells may effectively exclude B cells from T zones, even in the absence of the follicular chemoattractant CXCL13. In general, lymphoid chemokines are less potent than inflammatory chemokines. Although this necessitates a high level of chemokine expression to attract cells, it also permits

a greater range of chemokine sensitivity among different responding cell populations. Competition with naïve B cells is important for the exclusion of activated and autoreactive anergic B cells from follicles (5). In the presence of competitor B cells, activated B cells become highly enriched in a narrow strip at the boundary between T zones and follicles, increasing the probability of successful interaction with helper T cells. Although activated B cells do not lose expression of CXCR5 or the ability to respond to CXCL13 (KMA and Eric Ekland, unpub. obs.), more subtle changes in their responsiveness to lymphoid chemokines may be adequate to redirect their homing.

$CD4^+$ T cells also change their homing properties during T cell-dependent antibody responses, acquiring the ability to home to follicles and participate in germinal center responses (6, 7). I found that *in vivo*-activated T cells upregulate CXCR5 and become responsive to CXCL13 with kinetics that mirror their entry into follicles (Chapter 3). At the same time, responsiveness to the T zone chemokines CCL19 and CCL21 are downregulated. These data strongly suggest that reprogramming of lymphoid chemokine responsiveness is responsible for T cell homing to follicles during T cell-dependent antibody responses. However, because $CXCL13^{-/-}$ and $CXCR5^{-/-}$ mice lack follicles, it has not yet been possible to determine whether CXCR5 is required for activated T cell homing to follicles. Preliminary studies using mixed bone marrow chimeras have supported a role for CXCR5 in T cell homing to follicles (unpubl. obs.). Also consistent with a requirement for CXCR5, it has recently been established that all T cells present within germinal centers in human tonsil express CXCR5 (8). Underscoring the

importance of follicular homing of T cells, this and two other reports showed that tonsillar CXCR5$^+$ T cells with an activated/memory surface phenotype, but not similar cells that do not express CXCR5, potently induce antibody production when cocultured with B cells (8-10).

Interestingly, germinal centers form in T zones in mice deficient for CXCL13 (Chapter 2) or CXCR5 (11). This surprising outcome may reflect the failure of activated T and B cells to enter B cell areas in the absence of CXCL13. These germinal centers are smaller in size, but do contain CD4$^+$ T cells and a dense network of CR1$^+$ FDC (Chapter 2 and (11)). Even in wildtype mice, expression of FDC markers is strongest and most dense within germinal centers (see also Appendix 1). This may be a result of the high level of LTα1β2 on germinal center B cells. I found that this expression was maintained even in CXCL13$^{-/-}$ mice, and consistent with previous results (12), Vu Ngo showed that CD40 ligation induces high levels of LTα1β2 on B cells (Chapter 2). Since T cells are the predominant source of CD40L in germinal centers, these data implicate T cells as necessary inducers of the formation of the specialized germinal center stromal environment (see Appendix 1). Further studies are needed to define the role that CXCL13 plays in directing B and T cell traffic during immune responses and in the formation and function of germinal centers.

Although many antigen-specific CD4$^+$ T cells downregulate CXCR5 as early as ten days after immunization (Chapter 3), a subset of memory CD4$^+$ and CD8$^+$ T cells also express CXCR5 (13, 14) and respond to CXCL13 (Chapter 3). To date, no clear function has been found for CXCR5 on memory T cells,

although it appears that CXCR5 is expressed on subsets of both "central memory" T cells that maintain expression of CCR7 and "effector memory" T cells that lose expression of CCR7 (9, 10). CXCR5 expression on memory $CD4^+$ T cells may provide them with increased speed in interacting with B cells to provide help for antibody recall responses, or may improve the efficiency or quality of interaction with certain classes of dendritic cells. Recently, a dendritic cell population that enters lymphoid follicles has been characterized (15), and these cells may also utilize CXCR5 to guide their homing (16). In addition, CXCL13 expression has been detected in some dendritic cell subsets, including dendritic cells in sites of autoimmune inflammation in a murine lupus model (17, 18). CXCL13 expression by other cells has been detected in a variety of chronic inflammatory diseases (reviewed in (19)). Thus, the lymphoid chemokine CXCL13 may act as an inflammatory chemokine in some cases, guiding memory T cells (and B cells) to effector sites. CXCR5 may also aid in memory T cell homing to lymphoid organs, especially in the case of $CCR7^-$ memory T cells that can no longer utilize CCL21 to enter lymph nodes via HEV. $CCR7^{-/-}$ and *plt/plt* mice have only modestly decreased numbers of B cells and memory T cells in lymph nodes and Peyer's patches, so both of these cell types must be able to use other receptors or alternate pathways to enter these organs (20, 21). Consistent with the possibility that CXCL13 may contribute to the recruitment of CXCR5-expressing B and T cells into lymph nodes, CXCL13 has been detected on small vessels proximal to or within lymph node follicles (10). However, $CXCL13^{-/-}$ mice that are also homozygous for the plt mutation did not have a

striking defect in B cell accumulation within lymph nodes (unpub. obs.), indicating that at least one other chemoattractant must be able to support B cell recruitment to lymph nodes.

In contrast to the apparent redundancy in the molecular control of B cell homing to lymph nodes, I found that B cell homing to body cavities is strongly dependent on CXCL13 (Chapter 4). B1 cell accumulation is defective in the peritoneal and pleural cavities, but not the spleen of $CXCL13^{-/-}$ mice. CXCL13 is expressed by peritoneal macrophages and by radiation-resistant cells in the omentum, and by following B1 cell homing in adoptive transfer experiments, I identified the omentum as a portal of B1 cell entry to the peritoneal cavity. This pathway to the peritoneal cavity has also been suggested to be used by neutrophils and macrophages in responses to proinflammatory stimuli that induce omental mesothelial cells to produce inflammatory chemokines such as IL-8 and MCP-1 (22-24). Importantly, B1 function in body cavity immunity and natural antibody production were also defective in $CXCL13^{-/-}$ mice, indicating that B1 cell homing to the body cavities is important for their immune function.

Like CXCR5-expressing T cells, B1 cells are more sensitive to CXCL13 than B2 cells (Chapter 3&4), and the selective accumulation of B1 cells (versus B2 cells) within the peritoneal and pleural cavities may be controlled by their increased sensitivity to CXCL13. B1 cells also express higher levels of CXCR5 than B2 cells, but the level of CXCR5 expressed on *in vivo*-activated and memory T cells is significantly lower than that of B cells. Therefore, other cell-intrinsic factors must contribute to the regulation of chemokine sensitivity. In any

case, adoptive transfer experiments established that the enrichment of B1 cells in body cavities can be partially explained by their efficient homing to these sites from the bloodstream. This behavior may depend on a high level of CXCL13 responsiveness, and it may also be influenced by differential expression or activation of adhesion molecules on B1 cells. The mixing of peritoneal B1 cells between parabiotic mice established that B1 cell homing to body cavities does occur in adult mice. However, the degree of B1 cell mixing was incomplete, confirming the long-held suspicion that B1 cells are relatively sessile within the peritoneal cavity, being retained there for periods of at least several weeks. In contrast, peritoneal B2 cells were mixed to equilibrium between parabiotic partners within eight weeks of blood exchange, revealing that B1 cells are selectively retained within body cavities. Nothing is known about the mechanism of B1 cell retention within body cavities or about the mechanism that allows B cells in lymphoid follicles to leave and re-enter the circulation. Future studies should address how retention and emigration from these areas of high chemoattractant concentration is controlled. Changes in chemokine responsiveness, differential adhesion, and competition for microenvironmental niches may all contribute to retention or emigration as they contribute to homing.

My studies also established a critical role for CXCL13 in the organogenesis of lymph nodes and Peyer's patches (Chapter 2). I hypothesized that CXCL13 may serve a similar function in lymphoid organ development as it does in follicle homeostasis (see also Appendix 2). Like follicular B cells, the IL7Rα^+ lymphoid tissue inducing cells (LTICs) that first colonize Peyer's patch

and lymph node anlagen express CXCR5 and deliver essential LTα1β2 signals (25, 26). Accentuating the relationship between follicle homeostasis and lymphoid organ development, it has recently been shown that LTICs establish follicular compartmentalization of Peyer's patch anlagen prior to the arrival of B cells (27). In addition, lymph node and Peyer's patch development in $CXCR5^{-/-}$ mice was partially rescued by a CXCR5 transgene that is expressed by LTICs, but not by B cells (unpubl. obs.). An important recent study by Honda *et al* established that CXCL13 is expressed in Peyer's patch anlagen and that LTICs respond to CXCL13 in chemotaxis assays (28). CXCL13 expression in Peyer's patch anlagen was reduced by treatment with an IL7Rα-blocking antibody *in utero*, suggesting that lymphotoxin signals downstream of IL7Rα are important for CXCL13 expression and LTIC recruitment (28). Though Peyer's patch organogenesis is clearly dependent upon an IL7Rα-dependent initiating signal, lymph node development is generally intact in $IL7R\alpha^{-/-}$ mice (and my unpublished data). Therefore, it is interesting to speculate that CXCL13 may be important for the induction of LTα1β2 expression by LTIC in addition to mediating their recruitment during lymphoid organ development. Redundancy between CXCL13 and IL7Rα ligands may account for the partial penetrance of defective lymph node and Peyer's patch organogenesis in $CXCL13^{-/-}$ and $CXCR5^{-/-}$ mice. However, additional chemokines and receptors likely also contribute to this process.

Secondary lymphoid organs have co-evolved with the increasing diversity of the lymphocyte antigen receptor repertoire and the advent of T cell-dependent

antibody responses. Primitive immune systems, such as those of sharks and rays, generate a smaller number of evolutionarily selected – instead of antigen-selected – antigen receptors, highlighting the fact that the adaptive immune system likely arose as a diversification of innate immune pathogen recognition mechanisms (29). The distribution of lymphocytes in these and other lower vertebrates is consistent with an emphasis on moving lymphocytes to sites of likely antigenic insult instead of recirculatory immune surveillance. Given the high level of exposure of the gut to environmental antigens, it is not surprising that gut-associated lymphoid tissues are found even in sharks and rays, as is the spleen, but lymph nodes are absent from primitive immune systems (29). The resulting reduced efficiency of immune surveillance may be effectively offset, however, by the increased frequency and strategic positioning of lymphocytes that can respond against common pathogens. Lymph nodes first appear in mammals, as do T cell-dependent antibody responses featuring affinity maturation and up to eight different antibody isotypes with specialized functions (29). Secondary lymphoid organs are essential to orchestrate immune surveillance and T-B interaction in mammals, enabling the potential of a tremendously diverse lymphocyte repertoire to protect against unexpected pathogens. Nonetheless, the twin strategies of tissue-specific immunity and the evolutionary selection of antigen receptors that recognize common (expected) pathogens have been maintained in mammals.

Several specialized populations of lymphocytes with a unique tissue distribution and a conserved, restricted antigen receptor repertoire have been

characterized in mice and humans. For example, natural killer (NK) T cells reside mainly in the liver and recirculate little, though they are also present in the spleen but not lymph nodes (30). The majority of NK T cells in mice and humans express a highly conserved stereotypical T cell receptor that confers CD1d-restricted reactivity against the glycolipid α-galactosylceramide (31). Likewise, particular immunoglobulin rearrangements are closely associated with specialized B cell populations, including B1 cells and marginal zone B cells (32-34). Consistent with the possibility that these invariant B cell populations are at least functionally related to primitive B cells that predate T cell-dependent antibody responses, both marginal zone B cells and B1 cells contribute mainly to T-independent antibody responses (34). A recent study using the R36A streptococcal vaccine demonstrated that both B1 cells and, to a lesser degree, marginal zone B cells contribute to responses against T-independent antigens that reach the spleen, whereas only B1 cells respond to a low dose of intraperitoneally injected antigen (35). The R36A vaccine is a bacterial extract, and the latter immunization may mimic intestinal injury, leakage, or invasion that would result in the release of bacteria into the peritoneal cavity, where they would be subject to the efficient antigen capture function of the omentum (22). Here, I have demonstrated that the peritoneal antibody response is reduced in $CXCL13^{-/-}$ mice, while the splenic response remains relatively intact (Chapter 4). These data indicate that CXCL13-directed B1 cell homing to body cavities, like the CXCL13-directed follicular homing of recirculatory B2 cells, is important for their immune function. These studies further suggest that the localization of B1 cells

within body cavities is an adaptation that places evolutionarily selected "useful" immunoglobulins in sites where they are most needed to control the pathogens to which they can respond. Overall, these studies establish CXCL13 as a central regulator of B cell homing and function in immunity.

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Appendix 1

Follicular stromal cells and lymphocyte homing to follicles

Cyster, J.G., K.M. Ansel, K. Reif, E.H. Ekland, P.L. Hyman, H.L. Tang, S.A. Luther, and V.N. Ngo. 2000. Follicular stromal cells and lymphocyte homing to follicles. *Immunol. Rev.* 176:181-193.

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Follicular stromal cells and lymphocyte homing to follicles

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Summary: Follicular dendritic cells (FDCs), the best defined stromal cell subset within lymphoid follicles, play a critical role in presenting intact antigen to B lymphocytes. The discovery that many follicular stromal cells make B-lymphocyte chemoattractant (BLC), a CXC chemokine that attracts CXCR5$^+$ cells, provides a basis for understanding how motile B cells come into contact with stationary FDCs. Here we review our work on BLC and discuss properties of BLC-expressing follicular stromal cells. We also review the properties of primary follicle and germinal center FDCs and suggest a model of FDC development that incorporates information about BLC expression. Finally, we consider how antigen recognition causes T and B lymphocytes to undergo changes in chemokine responsiveness that may help direct their movements into, or out of, lymphoid follicles.

Introduction

A striking feature of secondary lymphoid organ anatomy is the organization of B- and T lymphocytes into separate zones (Fig. 1). Existence of these zones was appreciated even before characterization of B and T lymphocytes, as they were defined by their thymus-dependence (T zones) or independence (follicles) (1, 2). Transfer experiments established that thymus-derived lymphocytes homed quickly into the thymus-dependent zones, while bone marrow-derived lymphocytes migrated to follicles (3). Another striking difference between these zones revealed in early studies was that injected antigens became distributed rapidly and broadly through follicular areas yet were not detectable in the thymus-dependent zones (4, 5). Other methods did reveal some antigen entering the thymus-dependent zones, but mostly inside cells of dendritic morphology (6, 7). These distinctions, of course, now fit well with current understanding of the different modes of antigen recognition by B and T lymphocytes, with B cells seeing intact antigen and T cells recognizing processed antigen as peptide–MHC complexes on bone marrow-derived dendritic cells. An important goal of present work is to put together the *in vitro* definition of lymphocyte antigen recognition with the *in vivo* anatomy, and define how lymphocytes come together so efficiently with the

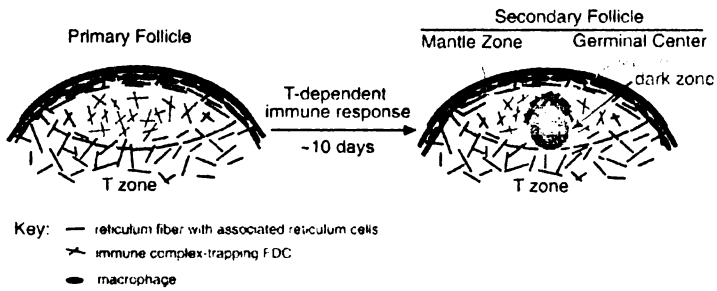

Fig. 1. Schematic illustration to show the relationship between primary and secondary follicles and their associated stromal cells. Primary follicles are predominantly composed of recirculating B cells (green shading), FDCs (red) and some non-immune complex trapping reticulum cells (black). T zones are predominantly composed of recirculating T cells and MHC class II$^+$ DCs (not shown) and fiber ensheathing reticulum cells (black). During an immune response, a germinal center may form within the follicle, converting it into a secondary follicle. Non-reactive B cells form the mantle zone around the germinal center. Antigen-specific B cell blasts (centroblasts) predominate in the germinal center dark zone (dark blue shading) and give rise to the centrocytes of the light zone (light blue shading). A complex network of FDCs occupies the light zone (red, thick lines). See (64, 111) for reviews on germinal centers. The follicles and T zone of lymph nodes and spleen are surrounded by a macrophage lined sinus (brown). Although not shown, macrophages are also present within germinal centers. In spleen, a specialized anatomical compartment exists across the sinus from follicles, termed the marginal zone, that is rich in macrophages and CR1/2$^+$ B cells (not shown, see (125)).

appropriate forms of their antigen inside lymphoid organs. A significant recent development in this area has been the discovery that small chemotactic cytokines, or chemokines, play critical roles in directing cell movements within lymphoid organs. Here we will not attempt to overview this topic but rather we will focus on our studies on B-lymphocyte chemoattractant (BLC), also called B-cell attracting chemokine-1 or BCA1, a molecule that helps guide cells to lymphoid follicles. For more general discussions on chemokines and cell migration, the reader is referred to several recent reviews (8–12).

B-lymphocyte chemoattractant (BLC)

BLC was discovered during the characterization of novel chemokine-related expressed sequence tags (13, 14). An 88 amino acid CXC chemokine, BLC has similarity to other CXC chemokines, including interleukin-8, interferon-inducible protein-10 and stromal cell-derived factor-1 (13). *In vitro*, recombinant BLC is an efficacious attractant of B lymphocytes, acting in transwell migration assays to attract larger numbers of naive B cells than any other defined chemokine (13, 14). All mature B cells express the BLC receptor, CXCR5 (formerly BLR1), and the *in vitro* studies are consistent with the notion that they can all respond to BLC (15, 16). However, not all CXCR5$^+$ B cells migrate to areas of BLC expression *in vivo*, as will be described further below, indicating that other factors can act to override BLC responsiveness and control B-cell positioning. CXCR5 is also expressed on some activated and memory T cells, and in addition to conferring BLC responsiveness (15, 17), this provides a co-receptor for HIV2 infection (18). The likely role of CXCR5 in directing movements of activated T cells will be discussed in a later section of this review. Although most myeloid cell types do not express CXCR5 or respond to BLC, a cDNA encoding a truncated form of CXCR5 that lacks most of the amino terminal ectodomain, termed MDR15, was isolated from human monocytes (19). Whether this form of CXCR5 is active as a BLC receptor remains unknown. Some evidence also exists for CXCR5 expression in monocyte-derived dendritic cells (20). In B-cell development, CXCR5 is not expressed on pre-B cells and is upregulated in the immature B-cell compartment in parallel with other B-cell maturation markers, including IgD and CD21 (15, 16). Upregulation of CXCR5 on maturing B cells coincides with their acquisition of a follicular homing ability (21).

Northern blot analysis of multiple mouse and human tissues revealed that BLC is highly expressed in all peripheral lymphoid organs while being expressed in few other tissues (13, 14). By *in situ* hybridization, a reticular expression pattern is observed throughout primary lymphoid follicles in spleen, lymph nodes and Peyer's patches (13, 22). No BLC was detected in purified B cells or T cells and expression in RAG1 $^{-/-}$ spleen, although reduced, was still significant (13). These combined observations established that BLC is produced by non-lymphoid cells resident in lymphoid follicles. In general histological terms, organs are described as consisting of parenchyma and stroma, where the parenchymal cells are the special-

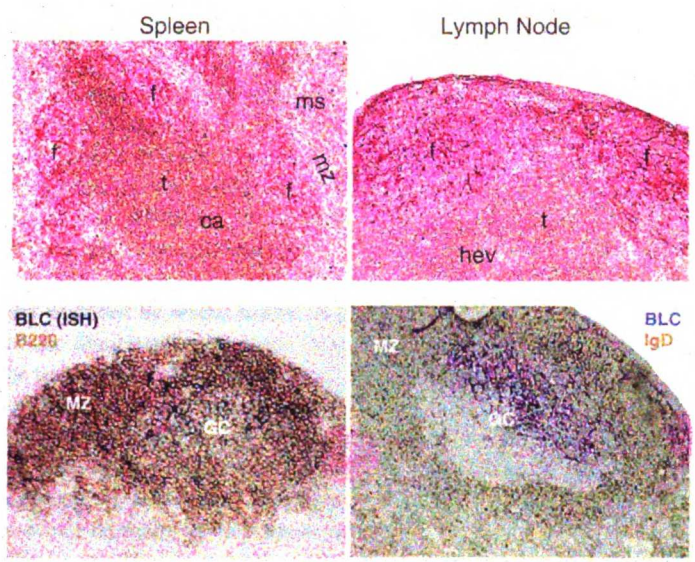

Fig. 2. BLC protein distribution in mouse spleen and lymph node Frozen sections were stained to detect BLC (red) and CD3 (brown) as described (126). f, follicle; t, T zone; ms, marginal sinus; mz, marginal zone; ca, central arteriole; hev, high endothelial venule.

Fig. 3. Distribution of BLC RNA and protein in secondary follicles of mouse lymph nodes. Left panel: frozen section in situ hybridized (ISH) with a riboprobe to detect BLC (black) and stained with an antibody specific for B220 (brown). Right panel: frozen section stained with polyclonal anti BLC (blue) and IgD (brown). GC, germinal center, MZ, mantle zone. The germinal centers can be identified as the area within the follicle that is B220 low (left panel) or IgD negative (right panel). Antibodies and staining were as described (126).

ized cells of the tissue (e.g. the lymphocytes) and the stromal cells make up the supporting tissue (23). BLC-producing cells may therefore be considered stromal cells. To examine the distribution of BLC protein, spleen and lymph node sections were stained with an anti-BLC antiserum (Fig. 2). Highest expression appears concentrated in a pattern similar to that previously observed for the RNA signal, but lower amounts of BLC can also be seen distributed throughout most parts of the primary follicle. No BLC staining was detectable within the $CD3^+$ T-cell area or on high endothelial venules (Fig. 2). Within the spleen, BLC staining was detected on cells lining the follicular side of the marginal sinus, a site where BLC RNA is expressed (16, 24), whereas no BLC RNA or protein was detectable across the sinus in the marginal zone (Fig. 2). Levels of surface CXCR5 expression may also be an indication of the amounts of BLC protein in an area since *in vitro* studies show that BLC causes CXCR5 modulation on B cells (16). By flow cytometry, follicular-phenotype B cells have lower surface CXCR5 compared to marginal zone-phenotype cells (16), providing additional evidence that there are greater amounts of BLC in follicles than marginal zones. Overall, the data suggest that the BLC protein gradient is steep, and possibly much of the protein is associated with the membrane processes of the follicular stromal cells.

In previous studies of secondary follicles we observed that cells expressing high amounts of BLC RNA were frequent in mantle zones but appeared to be present in reduced numbers within the central regions of germinal centers (17). To further explore the distribution of BLC-expressing cells in secondary follicles, we analyzed sections of spleen and lymph node from immunized mice. Cells that hybridized strongly to the BLC riboprobe were frequent in the mantle zone, and positive cells could also be identified in the T-zone-distal region of the germinal center (Fig. 3A), most likely corresponding to the germinal center light zone (see Fig. 1). Few or no BLC-positive cells were identified in the T-zone-proximal (dark zone) region of germinal centers in lymph node or spleen, although occasional BLC-expressing cells were observed in the rim of cells separating the germinal center from the T zone (Fig. 3A). Immunohistochemical analysis for BLC protein showed a similar polarized distribution, with strong staining in the T-zone-distal (light zone) region and little or no protein in the T-zone-proximal (dark zone) region (Fig. 3B). Consistent with a functional role for BLC within germinal centers, it was recently reported that germinal center structures in CXCR5-deficient mice are disorganized (25). Our findings suggest BLC may be required for normal light zone/dark zone polarization of germinal centers and we are currently investigating this possibility in BLC gene-targeted mice.

Follicular stromal cells

In addition to its functional importance, BLC provides a new marker for studying the development and properties of follicular stromal cells. Although much has been learnt about the phenotype and function of follicular stromal cells (see (26) for a review), the pathway of their development is still not well

understood compared to other cells of the immune system (27, 28). This is due at least in part to the difficulty of studying the cells *in vitro*. Protocols have been developed for isolation of a major subset of follicular stromal cells, the follicular dendritic cells (FDCs) (29), but it has generally been difficult to obtain large numbers of cells at high purity. Furthermore, when placed *in vitro* the cells undergo rapid phenotypic changes. As a result of these difficulties, FDCs continue to be defined as 'cells identified in sections of lymphoid follicles that can trap and retain immune-complexes' (30). Stromal cells with these properties had originally been described by several names, including reticulum cells, reticular fibroblasts and dendritic reticulum cells (4, 31–34). The term FDC was introduced to help distinguish these cells from a cell of dendritic morphology present in T zones, the interdigitating dendritic cell (DC) (30). In the discussion below, we will use information about BLC to further describe the properties of follicular stromal cells. We will begin by describing reticulum fibers, the structural elements that many follicular stromal cells ensheathe, and will then consider the ultrastructural and molecular features of the cells before discussing current understanding of follicular stromal cell and FDC development.

Reticulum fibers

Most organs in the body have a structural supporting skeleton of collagenous fibers (23). In lymphoid organs and other highly cellular tissues, such as liver, the fibers form a branched network and consist predominantly of collagen type III (35). Also known as reticulum fibers (where 'reticulum' derives from the Latin word *rete* meaning net), they were identified historically because of their specific histological staining with silver salts (23, 35, 36). Other extracellular matrix molecules are often associated with reticulum fibers, including elastin, which helps provide elasticity to the organ, laminin, vimentin and fibronectin (35, 37–40). In lymph nodes, reticulum fibers form a network that interconnects with the collagenous capsule of the node (41, 42). The density of fibers in follicles is lower than in T-cell areas, a feature that suggests a different pattern of cell migration within the two zones (41, 42).

Reticulum cells and dendritic reticulum cells

Connective tissue fibers are made predominantly by fibroblasts (23) and this is thought to be the case also for the reticulum fibers of lymphoid organs (34, 43). A unique feature of the reticulum fibers in lymphoid organs is that they are completely ensheathed by cytoplasmic processes of the associated fibroblasts (31). These 'reticular fibroblasts' or 'reticulum cells' are stellate in shape, having an ovoid nucleus and long cytoplasmic processes (34). In early electron microscopy studies, primary follicles were described as containing only two cell types, lymphocytes and reticulum cells (5). Typically, reticulum cells contain few lysosomes and show little evidence of phagocytic activity, while often containing rough endoplasmic reticulum and well developed Golgi networks (5, 44–46), consistent with the possibility that they are active in protein (e.g. chemokine) secretion. Reticulum cells make contact with each other through their processes and in some cases form very tight adhesion contacts, or desmosomes (43, 47). Careful inspection of lymph node sections revealed only very few places where reticulum fibers were exposed and made contact with cells other than reticulum cells (48). Possibly the earliest evidence that the reticulum cells in primary follicles are distinct from similar cells in T-cell areas was the discovery that many of the follicular cells exhibit immune complex-trapping activity (4, 5). Electron microscopic analysis of antigen-trapping cells in primary follicles indicated that the antigen-labeled dendritic processes were often distinct from the cytoplasmic extensions which ensheathed collagen fibers (4). The presence of dendritic processes in addition to the processes ensheathing the collagen fibers may help explain why the network of cells within lymphoid follicles often appears to be as extensive as in T-cell areas (49, 50), despite the lower density of reticulum fibers (41, 42). It is also possible that some of the dendritic processes emanate from cells distinct from reticulum cells and this possibility will be discussed further in the next section.

Electron microscopical characterization of germinal centers revealed notable morphological differences in the reticulum cells compared with cells in mantle zones (Fig. 1), leading to their being termed dendritic reticulum cells (4, 34, 44). These differences were most apparent within the germinal center light zone, where the cells demonstrated numerous membrane folds that formed a labyrinth structure (34, 47). Dendritic reticulum cells typically were not found to ensheathe collagenous fibers (34). Despite these differences, the reticulum cells in germinal centers and primary follicles bear many ultrastructural similarities, and in several studies where development of germinal centers was followed over time, transitional forms between reticulum cells and dendritic reticulum cells were observed (34, 44, 51). Most ultrastructural studies have therefore favored the conclusion that dendritic reticulum cells of germinal centers develop from the reticulum cells of primary follicles. However, Tew and co-workers provided evidence that some of the dendritic reticulum phenotype cells may derive from monocytic antigen-transport cells that migrate to the nascent germinal center and develop a dendritic morphology (52).

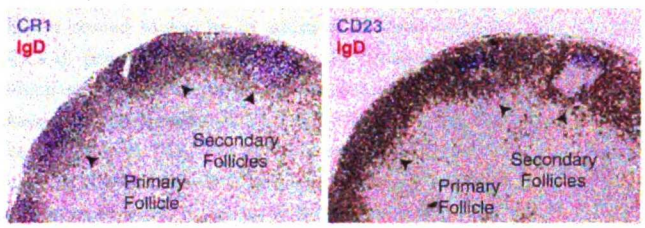

Fig. 4. FDC networks of primary and secondary follicles. Serial sections of lymph node were stained to detect IgD (brown) and either CR1 or CD23 (dark blue). Sections were counterstained light blue with hematoxylin. An extensive network of CR1$^+$ FDCs can be seen in both primary and secondary follicles (including the germinal center light and dark zones), whereas CD23$^+$ FDCs are restricted to germinal center light zones in secondary follicles.

Follicular dendritic cells of primary and secondary follicles

Since many follicular reticulum cells appear able to trap and retain immune complexes, they may be classified as FDCs (30). A lineage relationship between reticulum cells in the central regions of primary follicles and the dendritic reticulum cells of germinal centers, and their common designation as FDCs, has been further supported by molecular studies. Both cell types stain for complement receptor (CR) 1/2 and can bind antigens coated in breakdown products of complement components C3 or C4 (47, 53). Antibody-blocking experiments and analysis of gene-targeted mice indicate that CR1 and CR2 are the key immune complex-trapping molecules of primary follicles (53, 54). BP3/BST1 is also detected on FDCs in primary follicles and in mantle zones and germinal centers of secondary follicles (24, 50). The BP3 molecule has been characterized as a glycosylphosphatidyl-anchored membrane protein of the NAD glycohydrolase family, although its function remains unknown (50). The WP1 antibody is reported to stain the same network of cells as detected by anti BP3 (49, 55). These antibodies also stain reticulum cells in the T zone and often the follicular and T-zone stromal cells appear interconnected, supporting the notion that they are developmentally related. Two anti-mouse FDC monoclonal antibodies, FDC-M1 and FDC-M2, also recognize FDCs in primary follicles and germinal centers. The molecules recognized by these antibodies have not yet been defined, and both antibodies stain various cells in addition to FDCs (56, 57). The adhesion molecules intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and mucosal addressin-cell adhesion molecule (MADCAM)-1, are widely expressed by FDCs (47, 58). Some studies of isolated FDCs have indicated that they express surface molecules typical of hematopoietic cells, including CD45 and MHC class II (59). However, *in situ* analysis has not supported these observations and it appears that FDCs may pick up these molecules from the closely associated lymphocytes (59). In particular, there is little evidence that FDCs internalize/degrade antigens or present antigenic peptides on surface MHC class II molecules.

Interestingly, the reticular network of cells in primary follicles stained by BP3 typically appears more extensive than the network stained by antibodies to CR1 and CR2 (24). Consistent with the CR1/2 expression pattern, immune complex-trapping activity is stronger in the center compared to the periphery of most primary follicles (shown diagramatically in Fig. 1) (6, 60, 61). The mechanism by which this variation arises between the center and periphery of primary follicles is unknown. Perhaps contact with marginal zone/marginal sinus macrophages and T-zone cells provides signals that antagonize development of primary follicle FDCs. Since FDCs are defined as immune complex trapping cells (30), the stromal cells at the periphery of many primary follicles cannot be termed FDCs (Fig. 1). Despite these differences, the cells in the center and periphery of primary follicles appear to share many markers and electron microscopical studies have not highlighted differences, favoring the notion that all follicular stromal cells are closely related. This possibility is also supported by BLC *in situ* hybridization, as we find that BLC is highly expressed in both central and peripheral (T-zone-distal) regions of primary follicles (Figs 2 & 3). Preliminary double-staining immunohistochemistry experiments show BLC protein on CR1$^+$ and CR1$^-$ cells (data not shown). However, given that so far it has not been possible to track the development of FDCs from defined precursors, it remains possible that the immune complex-trapping cells in the center of follicles are distinct from the reticulum cells. In this case, some of the markers described above as staining FDCs might actually be staining non-immune complex trapping reticulum cells and, reciprocally, the reticulum cells might not express some markers such as CR1. We consider this possibility unlikely, but the inability to rule it out most likely reflects several parameters, including the following: the minimal number of *in situ* co-staining experiments that have been performed; the difficulty of distinguishing *in situ* between co-expression of molecules by the same cell, expression by overlapping cells or pick-up of molecules from adjacent cells; and the difficulty of analyzing marker expression at the single cell level on freshly purified stromal cells. Future studies must develop methods to address these issues.

In contrast to primary follicles, all the stromal cells within germinal center light zones are thought to be immune complex

trapping and therefore fit the definition of FDCs. These cells display several molecules in addition to those expressed by FDCs of primary follicles (Figs 4 & 5). These include FcγRs, which confer the ability to bind immune complexes even in the absence of complement (47, 53, 62), CD23, a low affinity IgE receptor (Fig. 4) (63, 64) and the molecules recognized by mAbs SKY01 and SKY49 (65). Light zone FDCs also appear to stain more intensely for many of the shared markers, including BP3 and CR1/2 (Fig. 4), although this might reflect the increased density of membrane processes rather than an increase in the number of molecules per unit area of membrane. As already mentioned, in situ hybridization and immunohistochemical staining for BLC indicates there is expression in the T-zone-distal (light zone) region of germinal centers but little or no expression in the T-zone-proximal (dark zone) region (Fig. 3). These observations suggest that some light zone FDCs, but few or no dark zone FDCs, may produce BLC. In future studies we hope to combine *in situ* hybridization for BLC with staining for FDC markers to determine directly whether BLC is made by light zone FDCs or whether these cells bind BLC protein that has been secreted by other stromal cells.

FDC development

In mice and rats, development of immune complex-capturing activity by stromal cells in lymphoid organs occurs between 2 and 3 weeks after birth (46, 60, 65, 66). This event depends critically on the presence of B cells but not T cells (67–69). However, the origin of the earliest FDC precursors is unclear. The fibroblastic morphology of FDCs, discussed above, favors the possibility of a mesenchymal origin (Fig. 5). FDCs are also thought to express several molecules typical of mesenchymal cells, including vimentin and desmin (70). Studies following FDC development at sites of chronic inflammation have also suggested they arise by local differentiation of mesenchymal cells (71, 72). When irradiated wild-type animals are reconstituted with bone marrow from allelically distinct donors, FDCs remain of recipient allotype further supporting a stromal, non-hematopoietic origin (54, 55, 73, 74). However, because FDCs are highly radiation resistant (75), it has been suggested that once they develop they might be difficult to replace using bone marrow transfer. In support of this, when FDC-deficient severe combined immunodeficient mice were irradiated and injected with large doses of total bone marrow or fetal liver cells, some donor-derived FDCs could be identified in the recipients after 4–6 months of reconstitution (76). While these results are consistent with a hematopoietic origin for some FDCs, bone marrow (or fetal liver) mesenchymal cells are likely to have been co-transferred with the hematopoietic progenitor cells, leaving open the possibility that the donor-derived FDCs were of mesenchymal origin. In addition to the ability of co-transferred bone marrow stromal cells to give rise to more bone marrow stroma (77–79), recent studies suggest that these cells can give rise to many other cell types, including muscle cells, astrocytes, endothelial cells and hepatocytes (80–83). Irrespective of the precise lineage of the progenitor cells, these transfer experiments provide evidence that some FDCs can derive from migratory precursors (Fig. 5). It remains unclear how these observations relate to the monocytic antigen-transport cells (see preceding section) reported in electron microscopy studies as migrating into lymphoid follicles (26). Recent studies have indicated that activated splenic marginal zone macrophages and lymph node subcapsular sinus macrophages can carry antigen into the center of follicles (84). In addition, DCs have been identified within follicles (85) and germinal centers (86) that are related to the MHC class II$^+$ DCs of the T zone. Some of these DCs express FcγRs and complement receptors and may be capable of transporting intact antigen (85–87). Perhaps they also express CXCR5 and migrate to follicles in response to BLC. It has not yet been tested whether these cell types can acquire the ultrastructural phenotype and radiation resistance typical of FDCs. However, the inefficient seeding of FDC networks by bone marrow-derived cells in transplantation studies contrasts with the ability to replace lymphoid tissue DCs and macrophages with cells of donor origin and argues against the notion that cells of hematopoietic origin migrate into follicles during the immune response to form FDCs.

In summary, we presently favor models where the majority of FDCs develop from reticulum cells of non-hematopoietic origin (Fig. 5). While many of these cells may develop locally (Fig. 5, model 1), some might also be recruited from other sites in the tissue or from circulation (Fig. 5, model 2). Germinal center FDCs may derive from primary follicle FDCs (Fig. 5) and they might also be induced to develop directly from more primitive precursors. During some types of immune response, hematopoietically derived immune complex-presenting cells may enter the follicle, but these are unlikely to make a substantial contribution to the FDC network.

LTα1β2 and TNF in FDC and follicle development

New insight into the molecular requirements for follicular stromal cell and FDC development has come from a series of studies in mice deficient in lymphotoxin (LT) α1β2 and tumor necrosis factor (TNF) (88). Mice lacking either of these cytokines fail to develop normal stromal cell networks (88), established by lack of MADCAM1 staining of sinus-lining cells,

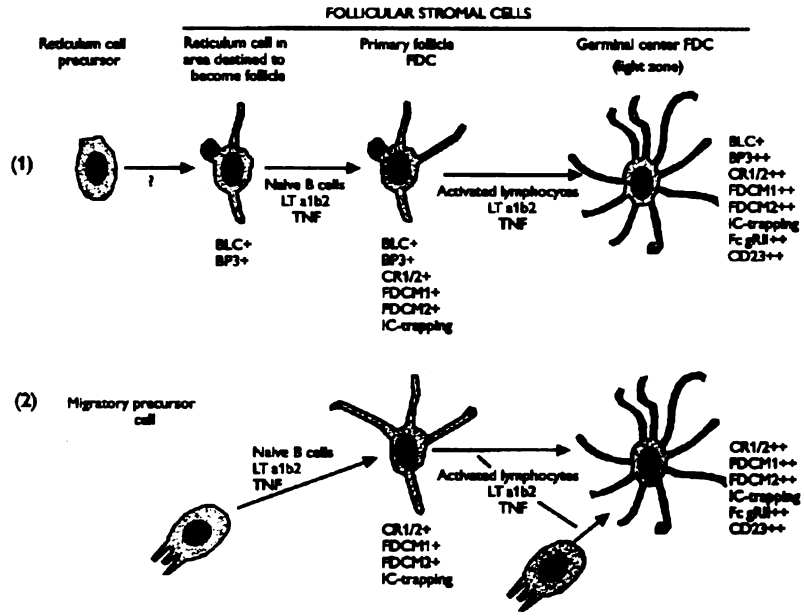

Fig. 5. Models of FDC development. In model 1, precursor cells of mesenchymal origin give rise to collagen fiber-ensheathing reticulum cells. Undefined signals cause reticulum cells in the area destined to become a follicle to express BLC. B cells recruited in response to BLC provide LTα1β2, TNF and possibly other signals needed for development of some of the reticulum cells into the antigen-trapping FDCs of primary follicles. BLC expression is essential for follicular clustering of B cells. Although it has not yet been demonstrated directly, the BLC in situ hybridization pattern is consistent with BLC expression by FDCs as well as by non-immune complex (IC) trapping reticulum cells at the follicle perimeter. Follicular clustering may be important in promoting B-cell encounters with FDC-associated antigen (5), and may play a role in promoting B-cell survival (21). During an immune response, further signals, likely provided by activated B cells and T cells, promote differentiation of primary follicle FDCs into germinal center FDCs. In cases where germinal centers form at ectopic sites, germinal center FDCs might develop directly from more primitive precursor cells. Light zone FDCs are distinguishable from FDCs of mantle zones/primary follicles and dark zones by high expression of CD23 and FcγRII and also by their more extensive membrane processes. BLC is expressed in germinal center light zones, most likely by FDCs, but is reduced or absent in dark zones. Germinal center FDCs provide signals that promote survival and proliferation of germinal center B cells (26). An alternative pathway of FDC development, shown in model 2, involves migration of precursor cells to the center of the primary follicle, or to the germinal center, where they differentiate and acquire a dendritic morphology. These cells might be immature reticulum cells or cells of a distinct lineage and they may be directly responsive to LTα1β2 and TNF or, alternatively, may depend on these cytokines for inducing molecules that help guide them to the central region of the follicle. These cells might not express BLC, BP3 or possibly some of the other markers listed. Future studies will need to track the development of FDCs from defined precursors to determine the relative contribution of these (or other) pathways to FDC development. These studies will also need to determine to what extent the various FDC markers are co-expressed versus being expressed by distinct cells in anatomically overlapping locations.

reduced or absent BP3 expression and lack of all FDC-specific markers (88). BP3 expression by stromal cells in T-cell areas is also disrupted (24). Therefore, the whole stromal network in the lymphoid area is dependent on signaling by LTα1β2 and TNF for normal development, further supporting a relationship between FDCs and other lymphoid tissue stromal cells. By immunohistochemistry, LTβ receptor (LTβR) and TNF receptor (TNFR) 1 are expressed on stromal cells, although further analysis is needed to establish whether all FDCs express both types of receptor (89, 90). In bone marrow transplantation experiments it has been established that FDC development requires LTα1β2 and TNF expression by radiation-sensitive cells LTβR and TNFR1 expression by radiation-resistant cells (91–95). These findings lend further support to the notion that FDCs are principally of non-hematopoietic origin (Fig. 5).

In each of the conditions where FDC networks fail to develop, polarized lymphoid follicles are not formed (88). Mice lacking the chemokine receptor CXCR5 (96) also fail to develop polarized follicles, leading to the speculation that LTα1β2 and TNF might be needed for normal expression of

CXCR5 ligands (97). This hypothesis was confirmed by the finding that BLC expression is reduced 20-fold in LT-deficient mice and 3–4 fold in TNF-deficient mice (24). Defective formation of lymphoid follicles in LT- and TNF-deficient animals can therefore be attributed at least in part to the reduced expression of BLC. Other molecules made by follicular stromal cells, such as the adhesion molecules ICAM1, VCAM1 and MADCAM1, are also likely to function in organizing cells in lymphoid follicles, and their importance in this process needs to be investigated. Interestingly, although BLC expression was markedly reduced in the cytokine and cytokine receptor mutant mice, it was not absent, even in LTα- and TNF-double deficient animals (24). In addition, BLC expression was also still detectable in animals lacking B cells (24). These findings suggest that the follicular stromal cell precursors present in lymphoid organs, perhaps a subset of the reticulum fibroblasts, are induced to express BLC by other as yet undefined developmental signals (*Fig.* 5). In this regard, it is significant that when B cells are transferred to B-cell-deficient recipients, animals that lack immune complex-trapping FDCs, the B cells migrate rapidly to the edge of the T zone and form polarized clusters (68, 98). The small amounts of BLC produced even before lymphocyte arrival and FDC maturation may be essential in initiating this B-cell clustering. As B cells are a critical source of LTα1β2 and TNF (88), this clustering is likely to promote establishment of a positive feedback loop that causes increased BLC expression, FDC maturation and further B-cell recruitment.

Signaling by TNFR1 and LTβR involves activation of TNFR-associated factor (TRAF), nuclear factor (NF) κB and NFκB-inducing kinase (NIK) family members, leading to the prediction that mutations in some of these downstream molecules may cause defects in lymphoid follicle development. This has been established by the observation that animals lacking NFκB family members p52/105 and bcl3 have defects in development of B-cell follicles (99–102). Recently, it was shown that these animals have diminished BLC expression (103). Adoptive transfer of wild-type bone marrow into p52-deficient mice failed to restore FDCs or follicles (103), consistent with the notion that NFκB functions in the LTβR signaling pathway within non-hematopoietically derived FDCs. The spontaneous mutant mouse *aly/aly* has a phenotype similar to LTα-deficient animals (104), and was recently shown to have a point mutation in NIK that prevents interaction with upstream mediator TRAF6 (105). The lack of FDC development in these animals (106) indicates they are likely to have diminished expression of BLC. Whether TRAF6 is needed in the pathway of FDC development and BLC expression has not yet been established because, in addition to lacking lymph nodes, TRAF6-deficient mice suffer perinatal lethality (107). Further investigation of the signaling events downstream of LTα1β2 and TNF should enhance our understanding of follicular stromal cell and FDC development. In addition to its significance for lymphoid organ development and function, this information will have importance in understanding the events that lead to development of FDCs at sites of chronic inflammation (71, 72, 108–110).

T-cell homing to follicles during the immune response

Germinal center formation is strongly dependent on T cells, with many studies providing evidence that T cells must migrate to the germinal center light zone and provide CD40L and other signals essential for centrocyte survival (64, 111). Migration of T cells to the boundary of B-cell areas early in the response may also be critical in favoring encounters between rare antigen-specific B and T cells. Substantial upregulation of CXCR5 was found to occur during CD4 T-cell activation *in vivo* with a time course consistent with CXCR5 helping direct activated T cells into follicles (17). Upregulation of CXCR5 only occurred under activation conditions that promoted T-cell migration to follicles. T cells typically become activated by encounter with antigen-presenting DCs in the T zone, where interactions may be promoted by the chemokines Epstein–Barr virus (EBV)-induced molecule-1 ligand chemokine (ELC) and secondary lymphoid organ chemokine (SLC) (11). In addition to increasing CXCR5 expression, T cells activated *in vivo* following immunization with antigen in adjuvant undergo a decrease in responsiveness to SLC and ELC (17). Several recent studies have established that decreased CCR7 expression is sufficient to cause T-cell exclusion from T-cell areas (112–114). Despite some unresolved issues, a model emerges in which appropriately activated CD4 T cells decrease responsiveness to SLC and ELC and as a result become excluded from the central T-cell area. At the same time, the cells increase expression of CXCR5. As the excluded cells approach the B-cell follicle they may enter the chemotactic gradient of BLC and become recruited into follicles. These changes in chemokine responsiveness may not be sufficient to direct T cells into B-cell areas, however, since in transfer experiments, T cells with this phenotype fail to migrate into follicles (17). Perhaps changes must also occur in adhesion molecule expression, for example changes that alter the relative affinity of the cells for other T cells (or features of the T-zone stroma) versus B cells (or FDCs). Further complexity arises when considering how T cells localize to germinal center light zones in preference to other parts of the secondary follicle. The involvement of additional guidance factors is strongly suggested.

Follicular exclusion of antigen-binding B cells

Engagement of B-cell antigen receptors above a threshold level causes B-cell exclusion from follicles and relocalization in the outer T zone (115). Follicular exclusion of foreign antigen-binding B cells is thought to help promote encounters with helper T cells during immune responses (116, 117). In the case of autoantigen-binding B cells in the Ig/hen egg lysozyme transgenic system, follicular exclusion is a means of tolerance induction that leads to elimination of the cells (21). Peripheral dsDNA reactive B cells are also excluded from migrating into follicles, and this may contribute to maintenance of B-cell tolerance to dsDNA (118). The observation that SHP1-deficient B cells become spontaneously excluded from follicles established that follicular exclusion is inducible by intracellular signaling in the B cell and is unlikely to be attributable to physical trapping by antigen (119). In an initial model, it was proposed that follicular exclusion might occur through diminished B-cell responsiveness to a B-zone attractant (115). However, in *in vitro* studies we have so far not found evidence of decreased BLC responsiveness in antigen-stimulated or anergic B cells (E. Ekland, J. G. Cyster, unpublished observations). Instead, we currently favor an alternative model that involves increased responsiveness to T-zone chemokines (120). Consistent with earlier findings that B-cell activation causes increased expression of CCR7 (formerly called EBV-induced molecule 1) (121, 122), acute antigen–receptor engagement was shown to cause increased *in vitro* responsiveness to ELC (120). Activated B cells also show increased responsiveness to SLC (V. N. Ngo, J. G. Cyster, unpublished observations), a second ligand for CCR7. Studies are ongoing to determine CCR7 levels on resting, activated and anergic B cells. In addition to changing receptor expression, antigen receptor signaling changes the expression of intracellular regulator of G-protein signaling (RGS) proteins, molecules that function as GTPase activators for heterotrimeric G-proteins (123). Characterization of mouse RGS1, RGS2, RGS3 and RGS14 expression demonstrated that *in vivo* BCR signaling leads to increases in RGS1 and 2 and decreases in RGS3 and 14 (124). *In vitro* transfection studies demonstrated that RGS1 and RGS3 are effective in downregulating signaling from several chemokine receptors, including the response to ELC and SLC (124). RGS14 is also thought to be a regulator of Gi and may also downregulate chemokine responses. Decreased RGS3 and RGS14 expression in activated B cells might therefore contribute to increased responsiveness to T-zone chemoattractants. T cells are not needed for follicular exclusion of antigen-binding B cells (98) and, consistent with the model, ELC and SLC expression is relatively unaffected in T-cell-deficient animals (V. N. Ngo, J. G. Cyster, unpublished observations). The finding that transferred CCR7$^{-/-}$ B cells pass more rapidly through the splenic outer T zone into follicles (112) provides evidence that responsiveness to ELC and SLC can influence the movement of B cells through the outer T zone. Future studies will need to investigate the migratory behavior of antigen-binding B cells in mice lacking CCR7, ELC or SLC.

General discussion

From the studies referred to above and from our on-going work on newly generated BLC-deficient mice (K. M. Ansel, J. G. Cyster, unpublished observations), it is now clear that the CXCR5-BLC receptor–ligand pair plays a critical role in organizing cells within follicles and influencing their development. The upregulation of CXCR5 that occurs on developing B cells is a critical aspect of B-cell maturation to a state competent for recirculation through lymphoid follicles. In future studies it will be important to test whether B-cell receptor signaling can influence the programed CXCR5 upregulation, and to determine whether CXCR5 upregulation is necessary for a B cell to become long lived. Future studies will also need to investigate the changes that occur in CXCR5^{+} follicular B cells that allow them to overcome the BLC-CXCR5 signal and leave the follicle to return to circulation. This information may be relevant to understanding the mechanism of antigen-induced follicular exclusion. In T cells, CXCR5 upregulation is likely to be important in helping direct activated helper cells into lymphoid follicles to support the germinal center response, although further work is needed to define precisely when CXCR5/BLC begins directing helper T-cell movements. The critical role of BLC/CXCR5 in follicular organization also makes it important to understand the factors regulating BLC expression. This includes determining whether LTα1β2 and TNF directly induce BLC or lead to upregulation indirectly, for example by promoting stromal cell proliferation or maturation. Future studies must also test whether BLC-producing stromal cells are precursors for primary follicle FDCs and whether these FDCs differentiate into BLC^{+} and BLC^{-} FDCs in germinal centers. In addition to their role in follicles, CXCR5 (96) and BLC (K. M. Ansel, J. G. Cyster, unpublished observations) play a role in lymph node and Peyer's patch development, and BLC expression has been reported at a site of chronic inflammation (22). Defining how BLC contributes to development of lymphoid organs and inflammatory lesions is likely to be an exciting area of future investigation.

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Appendix 2

Chemokines in lymphopoiesis and lymphoid organ development

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Chemokines in lymphopoiesis and lymphoid organ development

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An important role has emerged for chemokines in regulating the distribution of progenitor cells during hematopoietic cell development. As well as recruiting cells, chemokines promote cell retention and cytokine expression. Furthermore, chemokines have been found to have an inductive function in secondary lymphoid organ development.

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Abbreviations

CLL	chronic lymphocytic leukemia
DC	dendritic cell
DN	double-negative
DP	double-positive
HEV	high endothelial venule
HPC	hematopoietic progenitor cell
ICAM-1	intercellular cell adhesion molecule 1
IL-7Rα	IL-7-receptor α-chain
LT	lymphotoxin
LTIC	lymphoid-tissue-inducing cell
PTX	pertussis toxin
sIg	surface-immunoglobulin
SP	single-positive
TRANCE	TNF-related activation-induced cytokine
VCAM-1	vascular cell adhesion molecule 1

Introduction

Lymphocyte development depends on interactions between differentiating progenitor cells and supportive stromal cells in primary lymphoid organs. Much has been learned about the adhesion molecules that help mediate these interactions and about the cytokines that promote growth and differentiation of progenitor cells. Until recently, however, little was known about the cues guiding progenitor cells to their supportive niches. Studies over the past few years have provided evidence that members of the chemokine family function as such cues.

Chemokines are small, mostly secreted, proteins and they are classified into four families — termed C, CC, CXC and CX3C — based on the number and spacing of cysteine residues in the amino-terminal part of each molecule [1•]. Chemokine receptors are members of the seven-transmembrane-receptor family and chemotaxis is signaled by members of the pertussis toxin (PTX)-sensitive $G\alpha i$ subfamily of heterotrimeric G proteins. In addition to promoting cell migration, chemokines are active in inducing integrin activation and some promote intracellular changes in cells that include induction of the respiratory burst and transcription of cytokines. In this review, we discuss recent work that reveals critical roles for CXCL12 (previously termed SDF1; see Table 1) and its receptor, CXCR4, in hematopoietic cell development within fetal liver and bone marrow and we provide an update on the ongoing quest to identify the chemokines that are critical in thymopoiesis. The last part of this review focuses on the discovery that chemokines play a necessary role in the development not only of the cells that recirculate through secondary lymphoid organs but also of these organs themselves.

Table 1

Chemokines and their receptors that are discussed in this review.

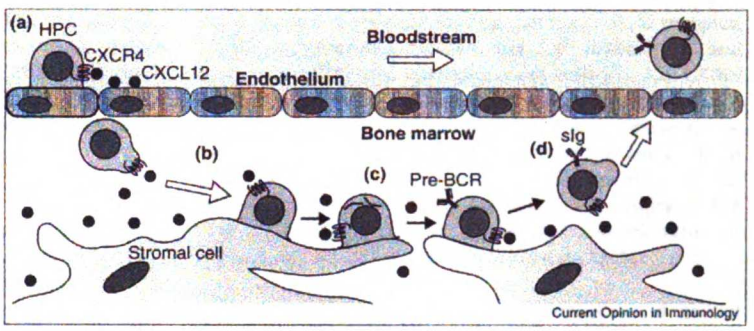
Systematic name	Common name	Receptor(s)
CXCL10	IP-10	CXCR3
CXCL12	SDF1	CXCR4
CXCL13	BLC/BCA-1	CXCR5
CXCL16	CXCL16	CXCR6
CCL2	MCP1	CCR2
CCL5	RANTES	CCR1, CCR3, CCR5
CCL11	Eotaxin	CCR3
CCL17	TARC	CCR4
CCL19	ELC/MIP3β	CCR7
CCL21	SLC/6-Ckine	CCR7
CCL22	MDC	CCR4
CCL25	TECK	CCR9
CX3CL1	Fractalkine	CX3CR1

We have utilized the new systematic names for chemokines [1•] throughout this review article. Common names or abbreviations are provided here and in the text for reference purposes as we adjust to this transition in chemokine nomenclature.

Stem cell homing and B cell lymphopoiesis: a central role for CXCL12

B cell lymphopoiesis is dependent on the microenvironment provided by the fetal liver and bone marrow. These organs contain stromal cells that provide growth factors required for B cell development. CXCL12, a chemokine produced by bone marrow stromal cells [2], was originally identified as a stimulatory factor for pre-B cells [3]. Mice that lack CXCL12 or CXCR4 die perinatally with cardiac, cerebellar, vascular and hematopoietic defects [4–6]. B cell lymphopoiesis and myelopoiesis are reduced in fetal liver and virtually absent in bone marrow of these mutant mice [4–6], and adoptively transferred $CXCR4^{-/-}$ fetal liver cells perform poorly in reconstitution of adult bone marrow B cell and myeloid precursors [7••,8]. Similar results were

Figure 1



Schematic representation of the proposed roles for CXCL12/CXCR4 in bone marrow B lymphopoiesis. Open arrows indicate cell movement; small arrows indicate developmental progression. **(a)** CXCL12/SDF1 may recruit CXCR4-expressing HPCs to the bone marrow by activating integrin-mediated firm arrest on vascular endothelium. **(b)** Stromal-cell-derived CXCL12 may direct hematopoietic cell homing within the bone marrow, promoting close contact between HPCs and supportive bone marrow stromal cells. **(c)** CXCL12 is required for efficient retention of developing pro-B cells (shown expressing cytoplasmic immunoglobulin heavy chain) and pre-B cells (shown expressing the pre-BCR) in the bone marrow and may also directly stimulate their growth. **(d)** sIg-expressing immature B cells downregulate their responsiveness to CXCL12, allowing their emigration out of the bone marrow into the circulation.

obtained when mice were reconstituted with bone marrow CD34+ hematopoietic progenitor cells (HPCs) transduced with a retrovirus encoding a CXCL12 intrakine that reduces CXCR4 surface expression [9].

The decreased numbers of B cell and myeloid precursors in CXCR4$^{-/-}$ fetal liver and reconstituted bone marrow are accompanied by the aberrant appearance of the precursors in the bloodstream, indicating that CXCR4 is necessary for retention of these progenitor cells within the fetal liver and bone marrow [7••]. In wild-type animals, B cells emigrate from the bone marrow into the peripheral circulation after they have reached the immature surface-immunoglobulin (sIg)+ stage. *In vitro* studies indicate that, as they reach this stage, they decrease their responsiveness to CXCL12 [10,11•,12•]. This change is likely to be critical in allowing the cells to escape the restraining activity of CXCL12 and exit to the periphery (Figure 1).

In addition to preventing precursor emigration, CXCL12 may direct close contact between HPCs and stromal cells within hematopoietic organs. For example, human fetal liver biliary ductal plate epithelial cells express CXCL12, and pre-B cells colocalize with this chemokine source [13]. As development progresses, biliary ductal plates regress in concordance with a decrease in CXCL12 expression and in liver hematopoiesis [13]. It will be interesting to see if there is a similar close association between HPCs and CXCL12-expressing stromal cells in the bone marrow and to determine whether this is disrupted when HPCs lack CXCR4.

Although the hematopoietic defects of CXCR4$^{-/-}$ and CXCL12$^{-/-}$ mice appear to be restricted to the B cell and myeloid lineages, CXCL12 and CXCR4 have been implicated in homing of more primitive HPCs to the bone marrow. Human CD34+ (multipotential) HPCs express CXCR4 and migrate in response to CXCL12 *in vitro* [14,15], and immobilized CXCL12 activates $\alpha_4\beta_1$-integrin-dependent adhesion of CD34+ HPCs on vascular cell adhesion molecule 1 (VCAM-1) under shear stress [16]. Consistent with a role in HPC recruitment to the bone marrow, pretreatment with blocking antibodies against either CXCR4 [17•] or $\alpha_4\beta_1$ [18] inhibited engraftment of human CD34+ HPCs in NOD/SCID mice.

HPC recruitment to the bone marrow is critical for engraftment in clinical bone marrow transplantation and may also be important during embryogenesis when hematopoiesis moves from the fetal liver to the bone marrow. CXCL12 expression is first detected in murine fetal liver during the period of colonization by HPCs (embryonic day [E]10.5–12.5) and falls off sharply as HPCs begin to emigrate from the liver to the bone marrow (E14.5) [19]. CXCL12 has been detected on human bone marrow microvessels [16] as well as stromal cells [2] and overexpression of human CXCR4 on mouse T cells caused accumulation of T cells in the bone marrow [20]. Thus, despite the ability of CXCR4-deficient stem cells to reconstitute lethally irradiated mice, these findings indicate that CXCR4 and CXCL12 may influence the efficiency of stem cell migration from the bloodstream into the bone marrow. A further test of the contribution of CXCR4 and CXCL12 to primitive stem cell homing might be to perform short-term adoptive transfer experiments with CXCR4-deficient HPCs. Should a role for this chemokine–receptor pair be confirmed, treatments that enhance CXCR4 function may be valuable as approaches to improve the efficiency of hematopoietic reconstitution by HPCs.

In addition to their role in normal hematopoiesis, CXCR4 and CXCL12 may also influence the distribution of chronic lymphocytic leukemia (CLL) cells. CLL cells invariably infiltrate the bone marrow of CLL patients and, in *in vitro*

studies, CLL cells were found to migrate beneath a layer of bone marrow stromal cells in a CXCR4-dependent manner [21]. CXCL12 might also influence CLL cell survival. *In vitro*, CLL cells rapidly undergo apoptosis unless they are cocultured with stromal cells and this effect was abrogated by CXCL12-blocking antibodies [22]. These studies suggest that blockade of CXCR4 or CXCL12 may have therapeutic potential in treatment of CLL.

Chemokines in thymopoiesis

Thymocytes undergo an orderly series of movements between thymic subcompartments as they develop into mature T cells. Cells of the early $CD4^-CD8^-$ (double-negative [DN]) stage are found in the subcapsular (outer cortical) region. Cells of the next developmental stage, the $CD4^+CD8^+$ (double-positive [DP]) cells, are predominant in the remainder of the cortex, where they undergo positive selection. Thymocytes that are selected move to the (central) medullary region while becoming mature $CD4^+CD8^-$ or $CD4^-CD8^+$ (single-positive [SP]) cells. Final maturation events take place in the medulla, including elimination of autoreactive cells by negative selection. All thymic compartments contain specialized epithelial cells that provide support to the tissue and also act as antigen-presenting cells. In addition, the medulla contains large numbers of antigen-presenting dendritic cells (DCs). Exit of SP cells from the thymus is thought to be via vessels at the cortico-medullary junction [23].

A role for chemokines in guiding cell movements within the thymus has been anticipated since the early finding that transgenic expression of PTX in thymocytes inhibits their exit from the thymus [24]. Many reports have since identified chemokine and chemokine-receptor expression in the thymus (see [25,26] for recent reviews). On the basis of strong evidence that chemokines play critical roles in organizing cells within peripheral lymphoid tissue subcompartments [27], it can reasonably be speculated that chemokines are needed to attract and/or retain cells within each of the thymic subcompartments.

Cortical expression in the thymus has been described for two chemokines, CXCL12 and CCL25/TECK [28•,29]. A role for cortical CXCL12 is supported by the finding that CXCR4 is expressed at high levels on DP thymocytes and that these cells are strongly responsive to CXCL12 [30•]. However, T cell development appears to occur normally in CXCR4-deficient embryos and in irradiated animals reconstituted with $CXCR4^{-/-}$ fetal liver cells [6,7••].

Coexpression of CD4 and CXCR4 by many thymocytes may allow their infection by HIV. The possible significance of this is highlighted by the observation that human fetal thymic transplants in SCID mice become depleted of thymocytes following infection by CXCR4-utilizing HIV variants [31].

CCL25 was initially detected within medullary DCs [32] but recent experiments indicate that it is also expressed by thymic epithelial cells and that expression can be detected in the cortex as well as medulla [29]. Expression in the cortex is consistent with the finding that the CCL25 receptor, CCR9, is expressed on DN and DP thymocytes, and CCL25 is efficient in attracting these cells [33–35]. CCR9 is downregulated as cells mature; late SP thymocytes are CCL25-unresponsive [30•,35]. CCL25 is also made in the fetal thymus, raising the possibility that it plays a role in recruiting thymic precursors [29,36•]. However, in an elegant *in vitro* analysis that investigated the ability of the fetal thymus to recruit thymic precursors, neutralization of CCL25 did not influence thymic seeding [36•].

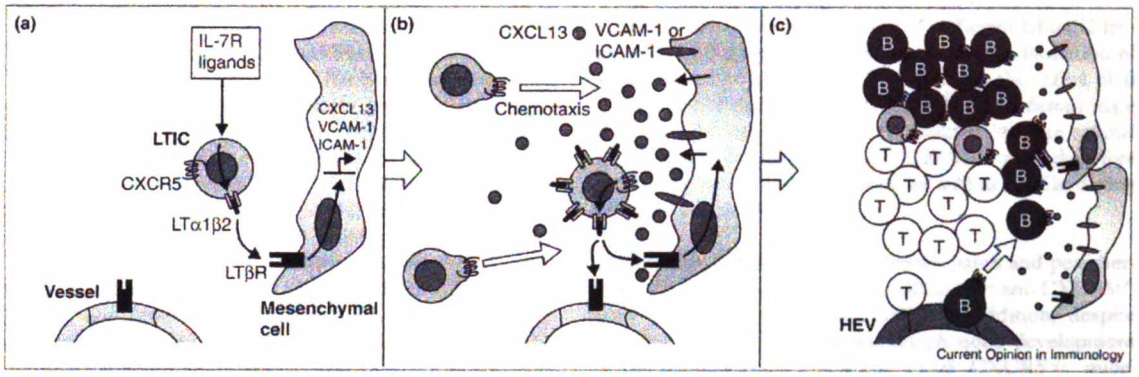
In contrast to the limited number of chemokines identified within the thymic cortex, multiple chemokines have been identified in the medulla, including CCL22/MDC, CCL17/TARC, CCL19/ELC/MIP3β, CCL21/SLC/6-Ckine [26], CCL11/eotaxin [37] and CXCL16 [38]. In immunohistochemical analyses of human thymus, CCL22 and CCL19 were found predominantly in nonoverlapping subsets of medullary thymic epithelial cells [39,40•]. Earlier studies in mouse thymus indicated that CCL21 is made by medullary epithelial cells and vessel-lining cells at the cortico-medullary junctions [41]. CCL17 has been found in thymic DCs [42]; in preliminary *in situ* hybridization studies, we observed expression restricted to the medulla (IIL Tang, JG Cyster, unpublished data).

Consistent with a role for CCL22 and CCL17 in attracting or retaining cells within the medulla, thymocytes upregulate CCR4 and become responsive to CCL22 (and presumably CCL17) transiently following positive selection and passage to the SP stage [30•,39,40•]. Upregulation of CCR7 occurs at a similar stage but expression of this receptor, and CCL19/CCL21 responsiveness, persists to the mature stage [30•,43,44]. DCs are also known to express CCR4 and CCR7 [45] although it has not yet been tested whether these molecules are expressed on thymic DCs.

The expression pattern of CCL19/CCL21 and CCR7 seems consistent with a role in organizing cells, possibly including DCs, within the medulla or in attracting cells to vessels at the cortico-medullary junction for exit from the thymus. Despite this compelling series of findings, an understanding of the role these molecules play in thymocyte development is still lacking. Analysis of CCR4-deficient mice [46], CCR7-deficient mice [47] and *plt/plt* mice (that lack CCL19 and most CCL21 expression [48,49]) has not revealed gross defects in T cell development.

Recently, a novel transmembrane chemokine, CXCL16, was identified that is expressed in the thymic medulla [38]. The CXCL16 receptor, CXCR6, was identified on $CD8^+$ SP cells and possibly some DN cells [38]. However, genetic studies have not revealed a limiting role for CXCR6 in T cell development [50]. Similarly, despite detection of the previously defined transmembrane chemokine, CX3CL1/Fractalkine, on elongated cells in

Figure 2



Proposed model for lymph node and Peyer's patch organogenesis. **(a)** IL-7Rα ligands initiate lymphoid organ development by stimulating surface expression of LTα1β2 on IL-7Rα$^+$CXCR5$^+$CD4$^{+/-}$CD3$^-$ LTICs. Mesenchymal cells expressing the LTα1β2 receptor (LTβR) respond by upregulating VCAM-1, ICAM-1 and CXCL13/BLC. **(b)** CXCL13 production by mesenchymal cells attracts additional LTICs and further stimulates LTα1β2 expression. This positive-feedback loop between CXCL13 and LTα1β2 creates a focal site of high CXCL13 and LTα1β2 concentrations, leading to coalescence of LTICs into focal organ anlagen. **(c)** Differentiation of local blood vessels into HEVs allows recruitment of B and T cells. B cells also express CXCR5 and respond to CXCL13; they may play a limiting role in Peyer's patch organogenesis and the formation of ectopic lymphoid tissues at sites of chronic inflammation. Panel (c) is drawn to a smaller scale.

the subcapsular region of human thymus [51], mice lacking the CX3CL1 receptor, CX3CR1, show normal T cell development [52].

In summary, although much has been learned about the chemokines and chemokine-receptors expressed in the thymus, we still know little about what regulates the movement of thymocytes from compartment to compartment or what controls local cellular interactions within subcompartments. That chemokines are involved is strongly supported by various experiments examining sensitivity to PTX [24,28•,36•]. Gene-targeting experiments have not revealed limiting roles for specific chemokines or their receptors in the transit of cells between subcompartments. However, these studies have not ruled out more localized effects of chemokines on cell–cell interactions. Such interactions may have a significant impact on the efficiency of thymocyte positive and negative selection events. In this regard, there is some evidence that the thymus contains a diversity of cells that express and present different peripheral antigens [53].

Perhaps differential chemokine expression helps ensure that developing thymocytes shuttle between the diversity of antigen-presenting cells, maximizing their exposure to various self-antigens. Chemokines may also help recruit peripheral cells to the thymus, as shown by the requirement of CCL11 for eosinophil accumulation in the thymic medulla [54]. Another interesting consideration is the extent to which chemoattractant signals compete with antigen-receptor signals. An elegant study of peripheral T cells recently showed that CCL19, CCL21 and CXCL10 could overcome an antigen-receptor signal and attract cells, whereas CCL22, CCL2/MCP1, CXCL12 and CCL5/RANTES could not [55•]. Perhaps chemokines such as CCL22 attract thymocytes into contact with thymic antigen-presenting cells whereas CCL19 functions to move the cells away after a period of antigen-receptor engagement. Obviously, much remains to be learned. In addition to further exploration of chemokine and chemokine-receptor expression in the thymus and tests for possible redundancy between family members, an analysis of the effects of chemokine/receptor deficiency on the efficiency of thymocyte positive and negative selection events is strongly needed.

Chemokines in lymphoid organogenesis

Once they have been released from the primary lymphoid organs, B and T lymphocytes recirculate through secondary lymphoid organs, including lymph nodes and Peyer's patches, in search of antigen. Significant insight has recently been gained into how these secondary lymphoid organs develop, including the demonstration of a requirement for the cytokine, lymphotoxin (LT)-α1β2, for the IL-7-receptor α-chain (IL-7Rα) and for the chemokine CXCL13/BLC/BCA-1. Studies have been most detailed in Peyer's patches, where an unusual population of small, motile hematopoietic cells has been identified that appears to induce the earliest stages of organogenesis. These cells are CD45$^+$CD4$^{+/-}$CD3$^-$IL-7Rα$^+$ and constitute a majority of cells present in Peyer's patch anlagen prior to colonization by B and T cells [56••].

Analysis of developing lymph nodes in fetal and newborn mice revealed the presence of a remarkably similar cell type [57,58]. Recently, two different lines of mutant mice

have been generated, one deficient for the transcriptional inhibitor, Id2, and another lacking the orphan steroid receptor, RORγ; in such mice these cells fail to develop and the animals lack all lymph nodes and Peyer's patches [59–61] (see also Update). Therefore, it is likely that organogenesis of Peyer's patches and lymph nodes proceeds via similar mechanisms induced by common (or closely related) 'lymphoid-tissue-inducing cells' (LTICs). Peyer's patch organogenesis has been dissected chronologically into three distinctive steps [62,63]. Firstly, induction of the adhesion molecules, VCAM-1 and ICAM-1 (intercellular cell adhesion molecule 1), occurs on intestinal mesenchymal cells. This step is dependent on IL-7R ligands and LTα1β2 and it does not occur in animals lacking LTICs [56••,59,64]. Secondly, coalescence of scattered LTICs at focal sites of VCAM-1/ICAM-1 expression and formation of the Peyer's patch anlagen take place. Thirdly, colonization of the anlagen by B cells and T cells occurs.

Gene-knockout experiments have revealed a role for CXCR5 [65] and its ligand, CXCL13 [66••], in lymphoid organ development. A detailed analysis of CXCL13$^{-/-}$ and CXCR5$^{-/-}$ mice showed that they lack most peripheral lymph nodes and Peyer's patches [66••]. In a reciprocal experiment, transgenic expression of CXCL13 in pancreatic islets was shown to promote, in an LTα1β2-dependent manner, development of ectopic lymph nodes [67•]. *In vitro* studies demonstrated that CXCL13 could directly induce LTα1β2 expression on resting B cells and CXCL13 was shown to function in lymphoid follicle development through the combined actions of B cell recruitment and induction of LTα1β2 on the recruited cells [66••]. As LTα1β2 promotes further expression of CXCL13 [68], this pathway creates a positive feedback loop that was suggested to be necessary for lymphoid follicle development. We propose a similar mode of action for CXCL13 in the development of lymphoid organs.

LTICs have been shown to express LTα1β2 [58,63], and IL-7Rα ligands can induce LTα1β2 expression on these cells [63]. However, the source of IL-7Rα ligands in the intestine is not yet clear. In a transgenic experiment, expression of IL-7 in intestinal enterocytes was found to enhance Peyer's patch formation in IL-7$^{-/-}$ mice, demonstrating that nonfocal production of IL-7 is sufficient to stimulate Peyer's patch development [69]. Therefore, IL-7Rα ligands are likely to initiate organ development by inducing LTα1β2 expression by scattered LTICs (Figure 2).

LTICs have also been found to express mRNA for CXCR5 [58,63]. Although the expression pattern of CXCL13 in the developing intestine has not been determined, it seems reasonable to speculate that expression begins at the same sites where VCAM-1 and ICAM-1 are expressed, as LTα1β2 can promote expression of all these molecules in other systems [68,70]. Once CXCL13 is expressed, it may gather LTICs into focal sites of organ formation, promoting integrin-mediated adhesion to VCAM-1- and ICAM-1-expressing mesenchymal cells and further inducing LTα1β2 expression (Figure 2). The focusing effect of the CXCL13/LTα1β2 feedback loop may nucleate a site of sufficient LTα1β2 levels to induce downstream events such as the formation of high endothelial venules (HEVs), allowing the influx of B and T cells (Figure 2). Interestingly, B-cell-deficient mice form reduced numbers of Peyer's patches, despite normal lymph node development [71]. CXCL13 may therefore function in recruitment of B cells, as well as LTICs, during Peyer's patch development.

Importantly, the disruption in Peyer's patch and peripheral lymph node organogenesis in CXCL13$^{-/-}$ and CXCR5$^{-/-}$ mice is not fully penetrant [66••]. In addition, despite depending on LTICs, mucosal lymph node development is not blocked in CXCL13$^{-/-}$ and CXCR5$^{-/-}$ mice. Although CCL21 can induce ectopic lymphoid tissue formation [72], mice lacking the CCL21 receptor, CCR7, develop a full set of lymph nodes and Peyer's patches [47]; these results imply that there is not a limiting role for these molecules in lymphoid organ development. Therefore, additional undefined chemokines/receptors are likely to participate in lymphoid organ development.

Ectopic lymphoid tissue formation is a feature of many chronic inflammatory diseases, including gastric lymphomas, autoimmune diabetes, Hashimoto's thyroiditis and Sjogren's syndrome. In recent studies, CXCL13 expression has been detected in the ectopic mucosa-associated lymphoid tissue induced by *Helicobacter pylori* infection of the gut, and CXCL13 is also present in the gastric lymphomas that occasionally develop in these patients [73]. Induction of CXCL13 and other lymphoid-tissue-inducing chemokines at a site of inflammation might convert the lesion from an acute to a chronic state. Blocking chemokine activity in chronic inflammatory diseases may therefore have significant therapeutic value.

Conclusions

In summary, chemokines are emerging as important players in lymphopoiesis and lymphoid organ development. CXCL12 plays a limiting role in retention of developing B cells and granulocytes within fetal liver and bone marrow and may also contribute to recruiting HPCs to these primary lymphoid organs. Multiple chemokines are made in the thymus, and developing thymocytes pass through stages of differential responsiveness to thymic chemokines, indicating that chemokines are likely to function in the fine control of cellular interactions within the thymus. Important advances have been made in the understanding of Peyer's patch and lymph node development, with strong evidence accumulating that a population of migratory hematopoietic cells are needed as inducers of secondary lymphoid tissue. The role of the CXCL13–CXCR5 chemokine–receptor pair in Peyer's patch and lymph node development is consistent with a requirement in recruiting these LTICs and promoting their expression of molecules, including LTα1β2, needed for lymphoid tissue development.

Update

In addition to LTα1β2, a second TNF-family cytokine, TNF-related activation-induced cytokine (TRANCE; also called OPGL, RANKL or ODF) and its receptor, TRANCE-R (also called RANK), are required for lymph node development, though Peyer's patch development is TRANCE/TRANCE-R-independent [74,75]. Recent work has demonstrated that LTICs express both TRANCE-R and TRANCE [76••]. Examination of rudimentary mesenteric lymph nodes of wild-type, TRANCE$^{-/-}$ and LTα$^{-/-}$ mice showed that the number and frequency of LTICs are decreased in both TRANCE$^{-/-}$ and LTα$^{-/-}$ mice [76••]. Importantly, the few LTICs present in these mutants fail to form the clusters observed in wild-type mice, instead being scattered throughout the rudimentary mesenteric lymph nodes. Transgenic overexpression of TRANCE restored LTIC cluster formation and most lymph node development in TRANCE$^{-/-}$ mice but had no effect on lymph node development in LTα$^{-/-}$ mice. Thus, clustering of LTICs is closely correlated with successful lymph node organogenesis, and the TRANCE/TRANCE-R and LTα1β2/LTβR pathways both play essential and nonredundant roles in this process. As both pathways may be upstream or downstream of chemokine signals, it will be interesting to dissect how these three sytems cooperate to regulate the movement of LTICs into clusters and ultimately the development of lymph nodes.

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Using TNF-related activation-induced cytokine (TRANCE)-knockout and -transgenic mice, this report demonstrates a correlation between LTIC (lymphoid-tissue-inducing cell) cluster formation in rudimentary mesenteric lymph nodes and the overall efficiency of lymph node organogenesis.

Appendix 3

Chemokines and B cell homing to follicles

Cyster, J.G., V.N. Ngo, E.H. Ekland, M.D. Gunn, J.D. Sedgwick, and K.M. Ansel. 1999. Chemokines and B cell homing to follicles. *Curr. Top. Microbiol. Immunol.* 246:87-92.

Chemokines and B-cell Homing to Follicles

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B cells that bind autoantigen in the periphery may be excluded from lymphoid follicles and rapidly eliminated (Cyster, 1997). To understand the basis for follicular exclusion we considered whether Gi coupled chemokine receptors might play a role by testing the effect of treatment with pertussis toxin (PTX), an inhibitor of Gi signaling, on B cell migration into splenic follicles. Strikingly, PTX treated B cells were unable to migrate into follicles or the white pulp cords of the spleen, whereas cells treated with buffer alone or with the oligomer B subunit of PTX could migrate into follicles normally (Cyster and Goodnow 1995). These observations led us to consider which chemokine receptors and chemokines might have a role in B cell positioning within lymphoid organs. We focused on two orphan receptors, BLR1 and EBI1, because these had been shown to be constitutively expressed by B cells in humans (Birkenbach et al. 1993; Dobner et al. 1992). To track expression of the mouse receptors, the amino-terminal ectodomains were expressed as GST fusion proteins and used to immunize rabbits. An antiserum against BLR1 was isolated and affinity purified using the same BLR1 fragment expressed as a fusion protein with mannose-binding protein. Flow cytometric analysis of mouse lymphoid tissues showed BLR1 expression on all mature B cells (Schmidt et al. 1998) with slightly higher surface expression on B cells with a $CD21^{hi}IgD^{lo}$ marginal zone phenotype (Fig. 1). BLR1 expression was also observed on $B220^{+}CD5^{+}$ peritoneal B-1 cells (Fig. 1). In B cell development, there was little or no BLR1 detectable on $B220^{+}IgM^{-}$ pro/pre-B cells, whereas $B220^{+}IgM^{+}$ immature B cells showed weak expression (Fig. 1; note that as BLR1 is detected with a polyclonal antiserum it is necessary to be cautious in interpreting the significance of weak signals such as seen on many of the cells in bone marrow gate G4). BLR1 expression became strongly upregulated on immature B cells at about the same time as surface IgD and CD21 (Fig. 1). The low expression by immature B cells is consistent with findings that immature B cells are inefficient at entering follicles (Cyster 1997) and suggests

that BLR1 upregulation may be an important part of the immature to mature B cell transition.

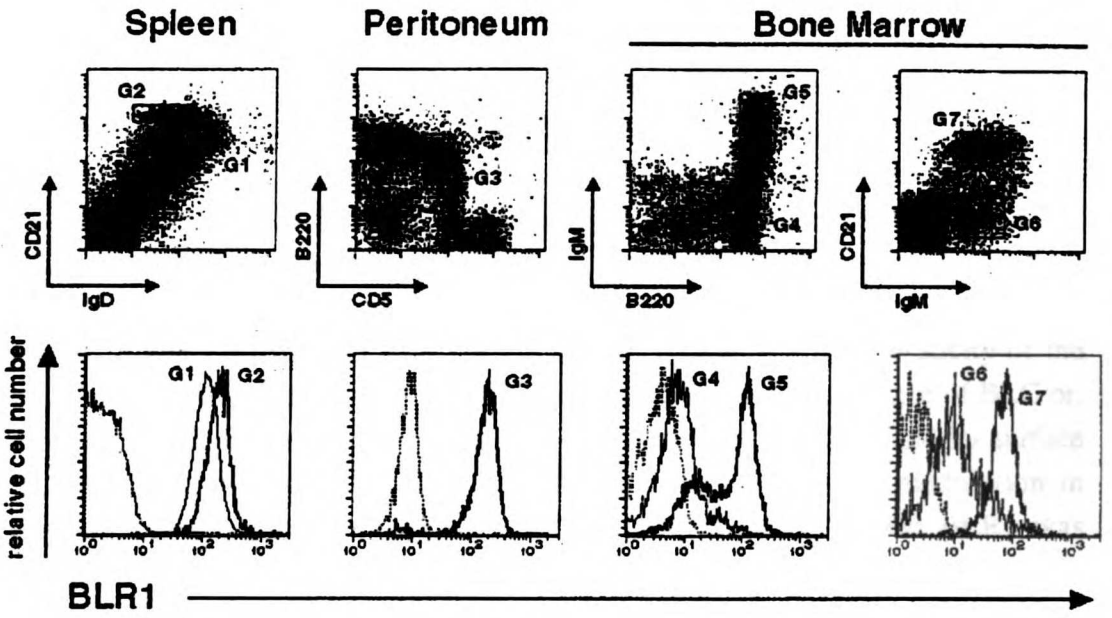

Fig. 1. Surface BLR1 expression by murine B lymphocytes. Cell suspensions prepared from the indicated tissues of C57BL/6 mice were stained with the antibodies shown in the upper panel and with rabbit anti-BLR1. BLR1 expression on cells within the gated regions in the upper panels is plotted in the lower panels. The gates correspond approximately to the following cell populations: G1, mature recirculating B cells; G2, marginal zone B cells; G3, peritoneal B-1 B cells; G4, pro/pre-B cells; G5, immature and mature bone marrow B cells; G6, immature bone marrow B cells; G7, mature recirculating bone marrow B cells. In the left and right-most panels, rabbit anti-BLR1 was detected with anti-rabbit-PE and in the center panels with anti-rabbit-FITC. The leftmost profiles in each histogram plot are samples stained without anti-BLR1 primary antibody as a control.

To allow identification and characterization of BLR1 ligands, BLR1 transfected 293 cells, Jurkat cells and E300-19 pre-B cells were generated. In situ hybridization experiments using novel expressed sequence tags (ESTs) related to chemokines as probes led to identification of one EST that hybridized to follicles in spleen, lymph nodes and Peyer's patches (Gunn et al. 1998). A recombinant form of the CXC chemokine encoded by this EST promoted calcium flux and chemotaxis responses in BLR1 transfected but not control cells, providing

evidence that it was a ligand for BLR1 (Gunn et al. 1998). Chemotaxis assays with freshly isolated cells showed that the mouse chemokine was an efficacious attractant of B cells, but attracted only small numbers of CD4 T cells and few or no macrophages or granulocytes (Gunn et al. 1998). These findings lead us to call the mouse chemokine and its human homolog, BLC for B-lymphocyte chemoattractant. In independent studies Legler et al., isolated the same molecule, termed it B-cell attracting chemokine (BCA)-1, and showed it was an efficacious attractant of human B cells (Legler et al. 1998). Based on these functional studies it has been concluded that BLC is a ligand for BLR1, leading to the renaming of BLR1 as CXCR5. Interactions between G-protein coupled receptors and their ligands frequently lead to modulation of surface receptor expression (Samanta et al. 1990; von Zastrow and Kobilka 1992). To further test the specificity of the BLR1/BLC interaction, splenic B cells were incubated in the presence of BLC or, as a control, IL-8, for 30 minutes at 37°C and then washed and stained for surface BLR1. Incubation with BLC, but not IL-8, lead to a two-fold modulation in surface BLR1 expression (Fig. 2). Kinetic analysis showed that BLR1 was modulated within 10 minutes of adding BLC. These results provide further evidence for the specificity of the BLC/BLR1 interaction and suggest that surface modulation may play a role in ligand mediated desensitization of this receptor.

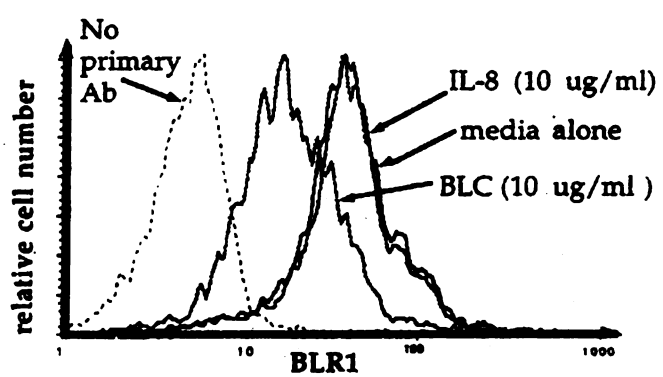

Fig. 2. Incubation with BLC induces internalization of BLR1. B cells were incubated in the absence or presence of chemokine as indicated for 30 minutes at 37°C and then stained on ice for BLR1.

In situ hybridization analysis demonstrated that BLC was highly expressed by cells with dendritic morphology in follicles (Fig. 3). Although BLC was not

detected in resting lymphocytes, lymphocyte deficient RAG1-knockout mice expressed 10-fold less BLC than wildtype controls (Fig. 3 and Gunn et al. 1998). Analysis of B and T cell deficient mice established that B cells but not T cells are needed for normal basal expression of BLC (Fig. 3 and Ngo et al. 1998). These findings are consistent with BLC being made by follicular dendritic cells, because maturation of these cells is B lymphocyte dependent (Yoshida et al. 1994). However, the expression pattern of BLC does not fully overlap with that of the FDC marker CD35. Pending further analysis on isolated cells, we refer to BLC producing cells as stromal cells since this designation encompasses FDC and other reticular cell types present in follicles. To further investigate the requirements for BLC expression by follicular stromal cells we studied mice deficient in TNF and lymphotoxin (LT) α/β. Mice deficient in TNF, LTα or LTβ lack organized follicles in the spleen (Matsumoto et al. 1997). The strikingly similar disruption of follicles in TNF-deficient mice and BLR1-deficient mice (Forster et al. 1996), suggested a genetic relationship between TNF and BLR1 (Goodnow and Cyster 1997). Analysis of BLR1 expression in TNF deficient mice and LTα deficient mice showed that BLR1 expression was not reduced but instead was slightly elevated (Cook et al. 1998). However, striking deficits were observed in expression of the BLR1 ligand, BLC, in these animals (Ngo et al. 1998). These reductions were readily apparent by in situ hybridization analysis (Fig. 3). When quantitated by Northern blot of total spleen RNA, an approximately three-fold reduction was detected in TNF-deficient animals and a 20-fold decrease in mice lacking LTα/β (Ngo et al. 1998). Expression of stromal cell derived factor 1 (SDF1) was not depressed in the mutant animals, providing some evidence for selectivity in the effect on BLC expression. Since BLR1 is required for normal organization of B cells in splenic follicles and since BLC is currently the only known ligand for BLR1, it is reasonable to propose that TNF and LTα/β promote lymphocyte compartmentalization in follicles by promoting BLC expression. Future studies must address whether TNF and LTα/β induce BLC expression directly or whether their role is indirect, promoting maturation of BLC expressing stromal cells.

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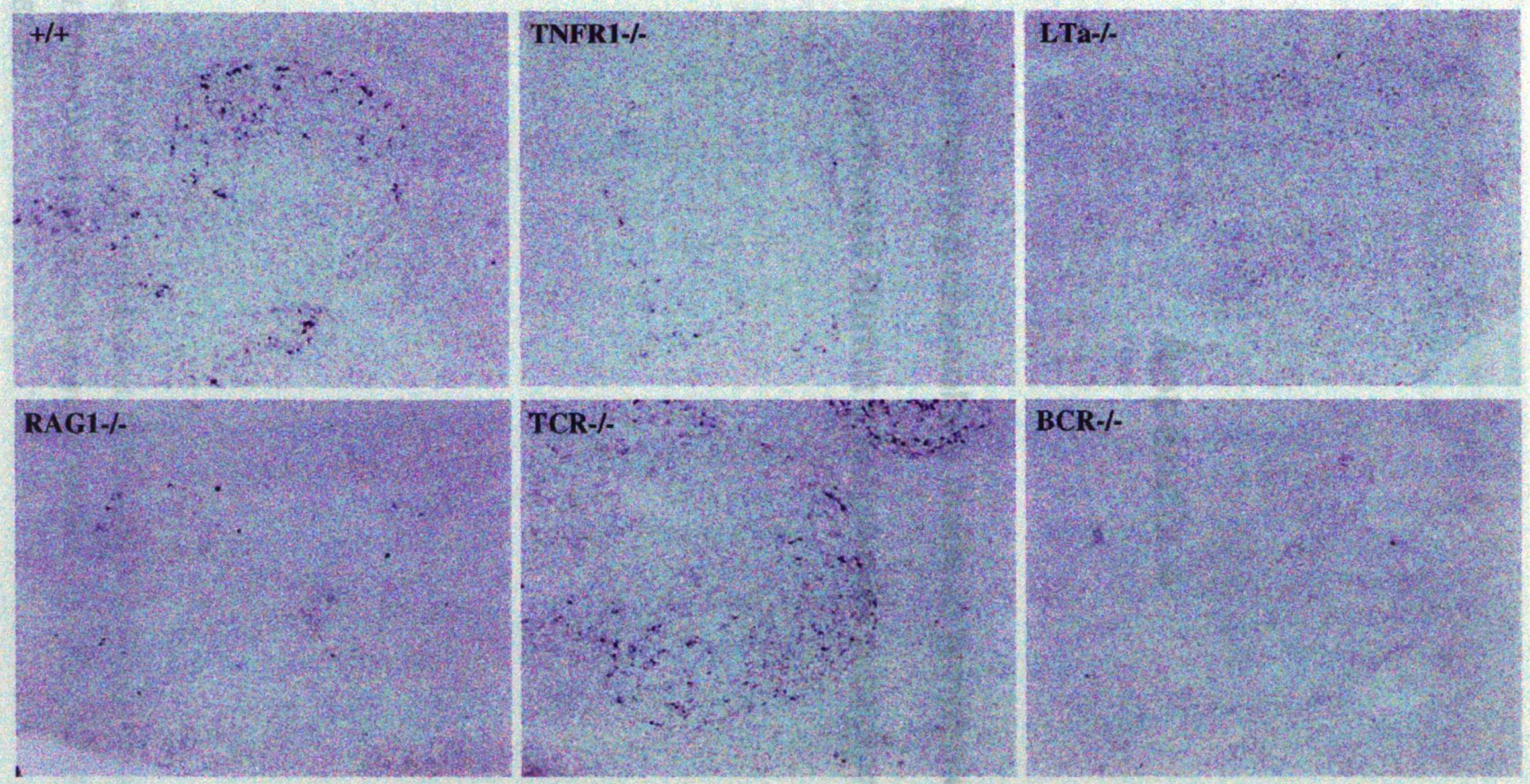

Fig. 3. In situ hybridization analysis of BLC expression in mouse spleen tissue from wildtype (+/+), TNFR1-/-, LTα-/-, RAG1-/-, TCRβ-/-δ-/- (TCR-/-), and μMT (BCR-/-) mice. Hybridization with the anti-sense BLC probe is seen as black staining. No signal was seen with a sense probe. Original magnification, 10x. In situ analysis was performed as described in Ngo et al., 1998.

Appendix 4

A B-cell homing chemokine made in lymphoid follicles activates Burkitt s lymphoma receptor-1

Cyster, J.G., V.N. Ngo, E.H. Ekland, M.D. Gunn, J.D. Sedgwick, and K.M. Ansel. 1999. Chemokines and B cell homing to follicles. *Curr. Top. Microbiol. Immunol.* 246:87-92.

A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1

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Secondary lymphoid organs (spleen, lymph nodes and Peyer's patches) are divided into compartments, such as B-cell zones (follicles) and T-cell zones, which provide specialized environments for specific steps of the immune response. Migration of lymphocyte subsets into these compartments is essential for normal immune function, yet the molecular cues guiding this cellular traffic are poorly defined. Chemokines constitute a family of chemotactic cytokines that have been shown to direct the migration of leukocytes during inflammation[1,2] and which may be involved in the constitutive homing of lymphocytes into follicles and T-cell zones[3–5]. Here we describe a novel chemokine, B-lymphocyte chemoattractant (BLC), that is strongly expressed in the follicles of Peyer's patches, the spleen and lymph nodes. BLC strongly attracts B lymphocytes while promoting migration of only small numbers of T cells and macrophages, and therefore is the first chemokine to be identified that is selective towards B cells. An orphan chemokine receptor, Burkitt's lymphoma receptor 1 (BLR-1), has been found to be required for B-cell migration into lymphoid follicles[6]. We show that BLC stimulates calcium influx into, and chemotaxis of, cells transfected with BLR-1. Our results indicate that BLC functions as a BLR-1 ligand and may guide B lymphocytes to follicles in secondary lymphoid organs.

To identify novel chemokines that might play a role in lymphocyte homing, we hybridized mouse tissues *in situ* with antisense transcripts of expressed sequence tags (ESTs) that showed homology to chemokines. One such EST (IMAGE Consortium Clone 596050) hybridized strongly to spleen, Peyer's patches and lymph nodes (Fig. 1d–f) but weakly or not at all to multiple non-lymphoid tissues (data not shown and Fig. 1l). We refer to this transcript and the protein it encodes as BLC. In the spleen, BLC hybridized to the B-cell-rich zones, or follicles, present in the outer region of the white pulp cords (Fig. 1d). A strong signal was detected in a reticular pattern within the follicle and at the outer boundary where the follicle meets the surrounding marginal zone (Fig. 1d, j). In Peyer's patches, expression of BLC was strongest within germinal centres, sites at which B cells undergo somatic mutation and affinity maturation[9], and extended into the surrounding mantle zone (Fig. 1f). Expression in lymph nodes was again concentrated in a reticular pattern within the follicles, but expression was variable and was not seen in all follicles (Fig. 1e, k). Northern blotting revealed a 1.2-kilobase (kb) transcript in wild-type spleen, Peyer's patches and lymph nodes, but not in resting B or T cells (Fig. 1l). BLC expression was reduced by 85% in spleens of lymphocyte-deficient *RAG1*-knockout mice (Fig. 1l), suggesting that lymphocytes provide a stimulus that promotes BLC expression in non-lymphoid splenic cells. Accumulation of follicular dendritic cells (FDCs) in lymphoid tissues depends on the presence of B and T lymphocytes[10]. Furthermore, FDCs have extensive processes that extend throughout lymphoid follicles in a pattern that is similar to the BLC *in situ* hybridization pattern[11]. These results indicate that FDCs may be a

source of this novel chemokine.

To identify the full-length complementary DNA encoding BLC, we searched for ESTs contiguous to the clone used for hybridization. Sequence analysis of four overlapping clones revealed a cDNA of 1,162 base pairs (bp), containing an open reading frame that encoded a putative protein of 109 amino acids with a predicted 21-amino-acid leader peptide (Fig. 2a). This sequence contained four cysteines in a pattern typical of the CXC family of chemokines[1]. BLC has most similarity to the CXC chemokine GROα (Fig. 2b). We also identified a cluster of six human EST clones encoding a protein with 64% amino-acid similarity to murine BLC. This protein is probably human BLC (Fig. 2b). A sequence-tagged site (STS) derived from this sequence (Genbank accession number G14456) has been mapped to chromosome segment 4q21 (ref. 12). This places the BLC gene near genes encoding most known CXC chemokines, including interleukin (IL)-8, GRO, IP-10, and PF4 (ref. 2). Interestingly, the protein with the greatest similarity to mouse and human BLC is Meq-sp, a product of the Marek's disease virus (MDV) Eco Q gene (Fig. 2b). MDV is a lymphotropic avian herpesvirus that causes a lethal disease in chickens. The disease is characterized by early cytolytic infection of B cells and subsequent development of T-cell lymphomas[13]. Meq-sp was identified in MDV-infected cells and was not previously recognized to contain a consensus chemokine motif[14].

These results indicate that BLC may be a B-lymphocyte chemoattractant. To test this possibility, chemotaxis assays were performed with lymphocytes from mouse spleen, using baculovirus-expressed BLC estimated to be >95% pure by silver staining. BLC induced a strong chemotactic response in B cells in experiments using either total spleen lymphocytes (Fig. 3a) or purified B cells (Fig. 3b), showing that BLC acts directly on B cells. In contrast, BLC showed limited activity towards T cells carrying CD4 or CD8 antigens (Fig. 3c, d). The B-cell response was chemotactic rather than chemokinetic, as cells incubated with BLC in the absence of a BLC gradient did not migrate (Fig. 3g). Stromal-cell-derived factor-1α (SDF-1α), previously described as the most efficacious chemokine for resting lymphocytes[15], attracted fewer B cells than BLC and lacked any B-cell specificity (Fig. 3a–d), highlighting the unique properties of this chemokine and leading us to name it as we have. BLC had weak but reproducible chemotactic activity for spleen monocytes/macrophages (Fig. 3e) but, in contrast to many CXC chemokines, showed no chemotactic activity towards granulocytes (Fig. 3f). Despite its efficacy as a B-cell attractant (Fig. 3a, b), BLC had a potency less than that of most chemokines, possibly because the baculovirus-expressed protein is not fully active. An alternative possibility is that a chemokine that is expressed constitutively within lymphoid tissues does not need to be highly potent.

All chemokines studied thus far signal via pertussis-toxin-sensitive G-protein-coupled receptors[2] and this is also true for BLC, as pertussis-toxin-pretreated B cells failed to migrate (Fig. 3h). Recent studies of mice with a targeted disruption of the orphan chemokine receptor BLR-1 indicated that this receptor is required for B-cell homing to follicles in spleen and Peyer's patches[6]. We therefore tested whether BLC could signal through BLR-1. Human embyonic kidney 293 cells stably transfected with mouse BLR-1 showed a dose-dependent calcium flux in response to BLC (Fig. 4a), whereas several other chemokines did not stimulate such a response and did not desensitize these cells to BLC (Fig. 4b). BLC did not stimulate a calcium flux in 293 cells transfected with the chemokine receptors CCR1, CCR2 or CXCR2, showing that the response of BLR-1-transfected cells to BLC was specific (Fig. 4c, d and data not shown). Using BLR-1-transfected 300-19 pre-B cells, a more complete dose-response curve was obtained; this showed that the response to BLC is saturable (Fig. 4e). Non-transfected 300-19 cells did not express detectable BLR-1 (Fig. 4g) and failed to respond to BLC (Fig. 4c).

We next tested the ability of BLC to stimulate chemotaxis through BLR-1. Jurkat T cells transfected with BLR-1 showed a chemotactic response towards BLC, whereas BLR-1-negative cells failed to respond (Fig. 4f). The maximum migration of transfected Jurkat cells occurred at a lower BLC concentration than was necessary for maximum migration of B cells, despite substantially lower expression of BLR-1 on the Jurkat cells (Fig. 4g). This may be due to our use of an amino-terminal epitope-tagged receptor in these cells, or

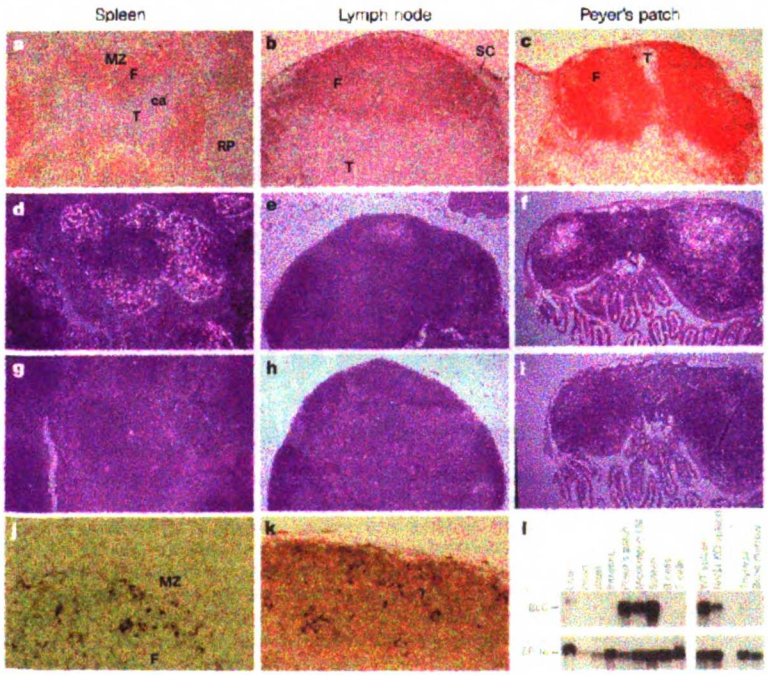
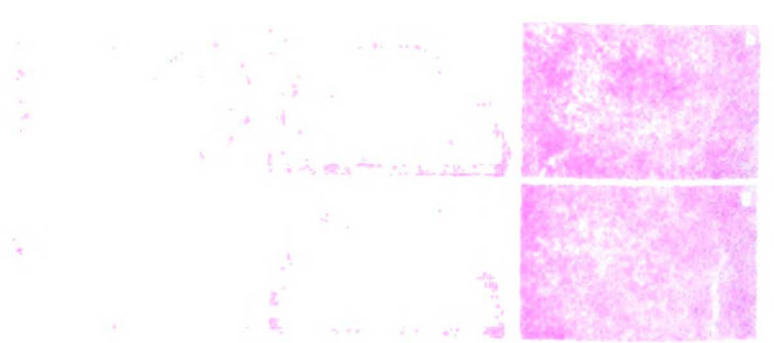


Figure 1 Expression pattern of BLC mRNA in mouse tissues. **a–c**, Identification of follicles by immunohistochemistry with anti-B220 is shown for orientation. Indicated structures are: ca, central arteriole; F, follicle; MZ, marginal zone; RP, red pulp; sc, subcapsular region; T, T-cell zone. **d–f**, Dark-field micrographs of hybridization of tissues with an antisense, ^{35}S-labelled BLC probe (2-week exposure). Signal is seen as white dots. **g–i**, Lack of hybridization to a ^{35}S-labelled sense BLC probe (8-week exposure). **j–k**, Bright-field micrographs showing hybridization with digoxygenin-labelled antisense BLC probe. Signal is seen as black staining. Original magnifications are ×10 (**a**, **c**, **d–f**), ×20 (**b**), and ×64 (**j**, **k**). **l**, Northern-blot analysis showing levels of BLC mRNA in the indicated cells and tissues. EF-1α hybridization indicates amount of total RNA loaded in each lane. KO, knockout; LN, lymph node; WT, wild type.



perhaps the coupling of receptors to downstream signalling pathways is different in mouse B cells and human Jurkat T cells. Mouse 300-19 pre-B cells transfected with wild-type BLR-1 showed a chemotactic response that had a similar dependence on the concentration of BLC to the response of purified B cells (data not shown). The chemotactic response of freshly isolated B cells, T cells, and Mac1$^+$ cells to BLC (Fig. 3) correlates with the reported expression of BLR-1 in all B cells and in subsets of CD4- and CD8-positive T cells[6,16,17] and with the expression of a variant form of BLR-1 in monocytes[16]. Our findings show that BLR-1 confers cells with responsiveness to BLC and that the responsiveness to BLC of cells from mouse lymphoid tissues correlates with the reported expression of BLR-1.

We have described a novel CXC chemokine, BLC, which is expressed in the follicles of the spleen, Peyer's patches and lymph nodes and is a strong B-cell chemoattractant. BLC's expression pattern, chemotactic activity, and ability to stimulate cells expressing BLR-1 suggest that it is a physiological BLR-1 ligand, acting to direct the migration of B lymphocytes to follicles in secondary lymphoid organs. Although BLR-1 is required for B-cell migration into splenic and Peyer's patch follicles, it is not needed for B-cell localization in lymph-node follicles[6]. This, together with our inability to detect BLC expression in all lymph-node follicles, indicates that other B-cell-specific chemokines may exist in lymph nodes. It is also possible that there are other BLC receptors that may function in lymph nodes. MDV, which is transmitted by inhalation but rapidly infects B cells[13], may have exploited the strong chemoattractant property of a BLC-like chemokine to attract target cells into

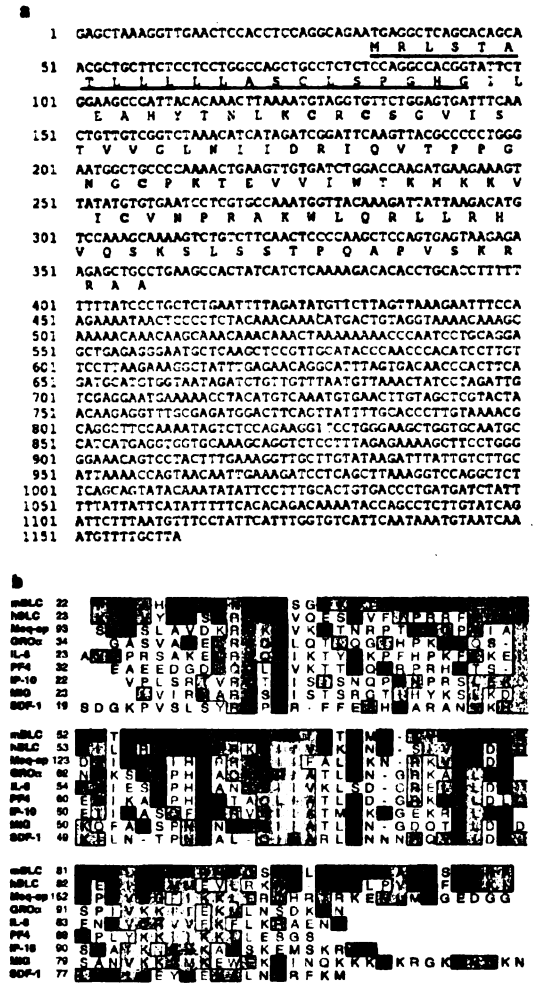


Figure 2 BLC sequence and alignment with other protein sequences. **a**, Nucleotide and deduced amino acid sequence of mouse BLC. The signal peptide, as determined by N-terminal sequencing, is underlined. Conserved cysteine residues are shown in bold. **b**, Alignment of the mouse (M) BLC protein sequence with those of human (h) BLC, GROα, IL-8, PF4, IP-10, MIG, SDF-1, and MDV-encoded Meq-sp. Identical amino acids are shaded dark grey. Conserved amino acids are shaded light grey. All shaded regions are boxed. Hyphens represent gaps inserted for optimum alignment. Numbers represent the position of the first amino acid shown in full-length protein.

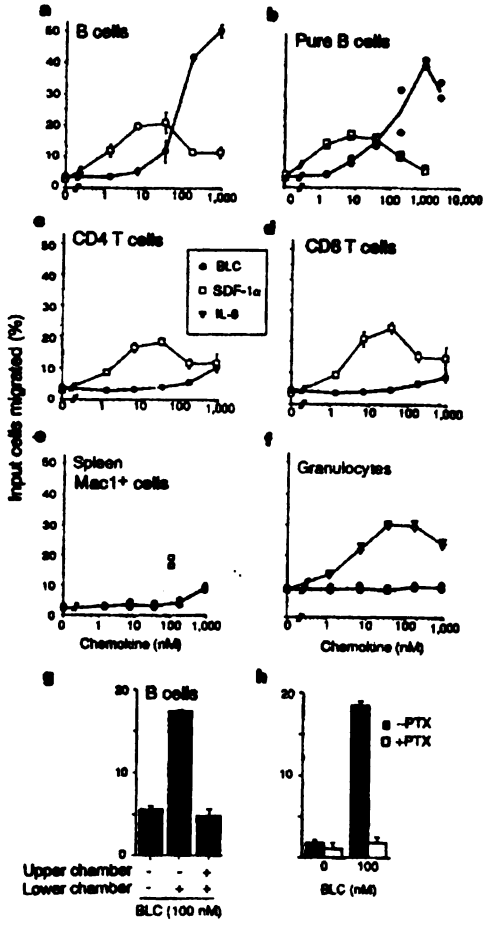


Figure 3 Chemotactic activity of BLC on leukocyte subtypes. Results are expressed as the percentage of input cells of each subtype migrating to the lower chamber of a transwell filter. Panels show migration of: **a**, B cells; **b**, purified B cells; **c**, CD4$^+$ T cells; **d**, CD8$^+$ T cells; **e**, monocytes/macrophages; **f**, granulocytes, towards BLC. Positive controls are SDF-1α (**a**–**e**) and IL-8 (**f**). **g**, Failure of B cells to migrate in the absence of a BLC gradient. BLC was added to the upper or lower chamber of the apparatus as indicated. **h**, Inhibition of BLC-induced migration by pretreatment of cells with pertussis toxin (PTX). Data points represent the mean ± s.d. for triplicates; individual data points are shown for duplicates. Each experiment was performed a minimum of two times.

the lung for infection. Our results and the identification of chemokines, expressed in lymphoid T-cell zones, that strongly attract resting T cells[19–21] indicate that chemokines may be the major cues promoting cell compartmentalization within lymphoid tissues. □

Methods

Sequence analysis. Pattern searches of the NCBI EST database using TFASTA[22], with human monocyte chemoattractant protein-1 as a template, retrieved human and mouse ESTs for BLC. BLAST[23] searches with these sequences identified contiguous ESTs. IMAGE Consortium [LLNL] cDNA clones 596050, 598232, 617961, and 749241 (ref. 24) were obtained from Genome Systems Inc. (St Louis, MO) as *EcoRI–NotI* inserts in the pT7T3-Pac vector, and sequenced. Similarity scores were calculated using the Blossum 30 matrix.

RNA expression studies. For northern-blot analysis, messenger RNA from mouse tissues or purified cells was subjected to gel electrophoresis, transferred to Hybond-N$^+$ membranes (Amersham), and probed using randomly primed mouse BLC EST 596050, which spans bases 10–532 of the BLC cDNA. For *in situ* hybridizations, paraffin sections (5 μm) from C57BL/6 mice were deparaffinized, fixed in 4% paraformaldehyde, and treated with proteinase K. After washing in 0.5 × SSC, the sections were covered with hybridization solution, prehybridized for 1–3 h at 55 °C, and hybridized overnight with sense or antisense ^{35}S-labelled riboprobe transcribed from the mouse BLC EST 596050. After hybridization, sections were washed at high stringency, dehydrated, dipped in photographic emulsion NTB$_2$ (Kodak), stored at 4 °C for 2–8 weeks, developed, and counterstained with haematoxylin and eosin. In some experiments, frozen sections were hybridized with sense or antisense digoxygenin-labelled riboprobes, immunostained with alkaline phosphatase coupled anti-digoxygenin antibody, and developed with NBT/BCIP as described (http://www.cco.caltech.edu/~mercer/htmls/Big_In_Situ.html). Immunohistochemistry with anti-B220 antibody was as described[5].

Production of recombinant proteins. The mouse BLC EST 596050 was cloned into the pVL1393 baculovirus transfer vector and cotransfected with BaculoGold (Pharmingen) into SF9 cells according to the manufacturer's instructions. For protein production, SF21 cells were infected at a multiplicity of infection of 10–20 and cultured in serum-free media for 60 h. Conditioned media was cleared, loaded onto a HiTrap heparin-affinity column (Pharmacia), and eluted with a 0.2–1 M NaCl gradient in 50 mM HEPES (pH 7.9). Fractions containing BLC were pooled, run on a C-18 reverse phase HPLC column (Vydac), and eluted with an acetonitrile gradient. SDS–PAGE and silver staining of this preparation showed a single protein band of the expected molecular mass for BLC (10K) that represented >95% of the total protein. Protein concentration was measured using the Bio-Rad protein assay. Protein-sequence analysis identified the isoleucine at position 22 as the amino terminus of the mature recombinant protein.

Chemotaxis. Lymphocytes and macrophages were obtained from spleens of C57BL/6 mice. For macrophage chemotaxis, B cells were depleted by passage over a MACS column (Miltenyi Biotec) after incubation with biotinylated anti-B220 antibodies and streptavidin-coated magnetic beads. Granulocytes were obtained from mouse bone-marrow suspensions. Mouse BLR-1-transfected Jurkat cells were obtained by transfection with pREP4 (Invitrogen) containing the mouse BLR-1-coding region[17], isolated by reverse transcription with polymerase chain reaction from mouse spleen RNA, and an N-terminal prolactin leader sequence and FLAG epitope[25] (provided by S. Coughlin). Positive clones were identified using anti-FLAG antibody M1 (Kodak). Chemotaxis assays were performed as described[19] using 10^6 cells per transwell and subsets of migrating cells were identified by flow cytometry using antibodies specific for B220, CD4 and CD8 (Pharmingen) and Mac-1 (Caltag, South San Francisco, CA). In some experiments, B cells were purified to >94% by MACS depletion of CD43$^+$ cells. Granulocytes were identified by their characteristic large side-scatter profile. The absolute number of each cell type added to transwells was: 5.2×10^5 B cells; 9.5×10^5 purified B cells; 1.7×10^5 CD4 T cells; 1.3×10^5 CD8 T cells; 3.8×10^5 granulocytes and 1.1×10^5 Mac1$^+$ spleen cells. In some experiments, cells were preincubated with 100 ng ml^{-1} pertussis toxin (List Biology Labs, Campbell, CA) for 2 h at 37 °C. IL-8 (R&D Systems) and synthetic human SDF-1α (N33A) synthesized by native chemical ligation (a gift from M. Siani) were used as positive controls. SDF-1α (N33A) has identical activity to native human and mouse SDF-1α (refs 26, 27).

Flow cytometry and calcium fluorimetry of transfected 293, Jurkat, and 300-19 cells. Native mouse BLR-1 was subcloned into plasmid vector pBK-CMV (Stratagene) and used to transfect HEK 293 cells and 300-19 pre-B cells (provided by T. DePranco). G418-resistant clones were tested for BLR-1 expression using an affinity-purified rabbit antiserum that is specific for the mouse BLR-1 N terminus (C. Timmons, C. Hsu and J.G.C., unpublished observations) and fluorescein isothiocyanate anti-rabbit secondary antibody (Caltag). HEK293 cells expressing CCR1, CCR2 and CXCR2 were provided by L. Charo. Calcium-mobilization studies were performed as described[28] using a Hitachi 4500 spectrometer. Intracellular calcium concentrations were calculated using the Hitachi 4500 intracellular cation measurement program.

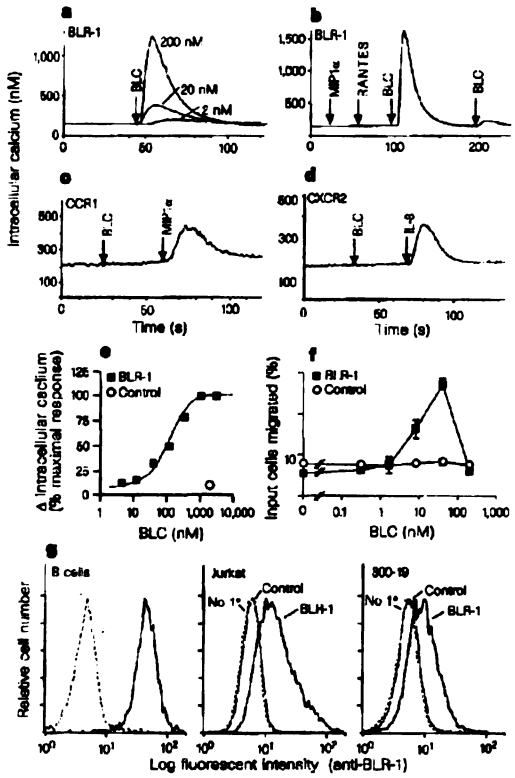


Figure 4 BLR-1 mediated calcium mobilization and chemotaxis in response to BLC. HEK 293 cells, transfected with the indicated chemokine receptors (a–d), were loaded with indo-1 and assayed by spectrofluorimetry for changes in intracellular calcium levels over time in response to BLC. a, Calcium flux as a function of BLC concentration (nM). b, Specificity of the response of BLR-1 to BLC. c, Lack of response of CCR1-transfected cells to BLC. d, Lack of response of CXCR2-transfected cells to BLC. e, Percentage of maximal calcium flux as a function of BLC concentration in BLR-1-transfected 300-19 cells. f, Chemotactic response of BLR-1-transfected Jurkat cells to BLC. Results are expressed as in Fig. 3. g, Flow-cytometric analysis of BLR-1 expression on spleen B cells and BLR-1-transfected (BLR-1) Jurkat and 300-19 cells. Non-transfected (control) cells and background fluorescence in the absence of primary antibody (No. 1°, dotted lines) are shown.

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The mouse and human BLC sequences are deposited in the Genbank database under accession numbers AF044196 and AF044197.

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