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Molecular basis of secondary lymphoid tissue compartmentalization

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Molecular Basis of Secondary Lymphoid Tissue Compartmentalization

by

Vu Nguyen Ngo

DISSERTATION

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DOCTOR OF PHILOSOPHY

in

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of the

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This dissertation is dedicated to my parents,

for giving me the best opportunity;

my brothers and sister,

for helping me to stay in focus;

and most importantly my wife,

Hanh Nguyen,

who has faith in me,

who encourages and supports

with her amazing patience and wonderful love.

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The co-authors in Chapter Two are Lucy Tang, who performed the RT-PCR experiments on purified cells, and Jason Cyster, who directed and supervised the research in this chapter as well as the rest of the experiments presented in this dissertation.

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PUBLICATIONS

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Ngo VN, Tang HL, Cyster JG.(1998). Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J. Exp. Med.* Jul 6;188(1):181-91.

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Ngo VN*, Ansel KM*, Hyman PL, Luther SA, Forster R, Sedgwick JD, Browning JL, Lipp M, Cyster JG. (2000). A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature.* 406(6793):309-14. * Equal first authors.

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Gunn MD, Ngo VN, Ansel KM, Ekland EH, Cyster JG, Williams LT. (1998). A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature.* 391(6669):799-803.

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Molecular Basis of Secondary Lymphoid Tissue Compartmentalization

by

Vu N. Ngo

ABSTRACT

Within secondary lymphoid tissues (spleen, lymph nodes, and Peyer's patches), immune cells are organized into two major compartments: B cell-rich follicles and T cell-rich areas. This cellular organization is thought to enhance antigen surveillance and to promote efficient encounters between rare antigen-specific lymphocytes. Previous studies identified lymphotoxin and the chemokine receptor BLR1/CXCR5 as important molecules for the generation of secondary lymphoid tissues and for cellular organization within these structures. The molecular mechanisms that regulate the formation and maintenance of these lymphoid compartments remained undefined. To address these questions, we hypothesized that the chemoattractive cues that are expressed in lymphoid compartments were essential in these processes. We identified a murine homologue of the chemokine ELC and showed that dendritic cells in the T zone constitutively express this chemokine. Recombinant ELC strongly attracted naïve T cells and activated B cells. These findings suggest a central role for ELC in both promoting encounters between

recirculating T cells and dendritic cells and in the migration of activated B cells into the T zone of secondary lymphoid tissues.

Mice deficient in TNF and lymphotoxin lack normal architecture in their secondary lymphoid tissues. We hypothesized that some of the defects in these animals were caused by defective expression of lymphoid tissue chemokines. We showed that expression of BLC, a B lymphocyte chemoattractant made by follicular stromal cells, was defective in these mice. Expression of the T cell attractant, SLC, by T zone stromal cells was also reduced. Treatment of adult mice with antagonists of LTα1β2 also led to decreased BLC and SLC expression. Furthermore, we found that expression of LTα1β2 on B cells is BLC dependent, and that recombinant BLC induces LTα1β2 upregulation on B cells *in vitro*. These results established the existence of a positive feedback loop between LTα1β2 and BLC, which is likely a mechanism for formation and homeostasis of lymphoid follicles.

Finally, we discovered that spleens from B cell-deficient mice have multiple defects in the T-cell compartment. We hypothesized that B cells and their LTα1β2 signals were necessary for the development of the splenic T zone. Experiments to test this hypothesis, including the generation of a lymphotoxin transgenic mouse model, demonstrated that LTα1β2 signals expressed by B cells were sufficient to promote

splenic T zone development. Practical and significant implications for human diseases from these studies will be discussed.

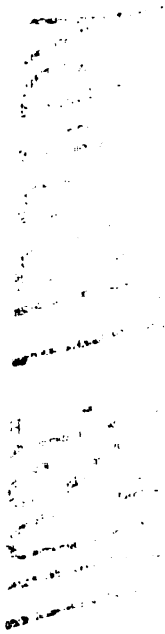

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BCR	B cell receptor
BLC	B lymphocyte chemoattractant
BLR	Burkitt's lymphoma receptor
CCR	CC chemokine receptor
DC	dendritic cell
DCCK	dendritic cell-derived CC chemokine
EBI-1	EBV-induced molecule 1
EF	elongation factor
ELC	EBI-1 ligand chemokine
ES	embryonic stem
EST	expressed sequence tag
FDC	follicular DC
HEV	high endothelial venules
LT	lymphotoxin
MAdCAM	mucosal addressin cell adhesion molecule
MCP	monocyte chemoattractant protein
MIP	macrophage-inflammatory protein
MMM	marginal metallophilic macrophage

MZM	marginal zone macrophage
PARC	pulmonary and activation-regulated chemokine
RAG	recombination activating gene
RT	reverse transcription
SDF	stromal cell-derived factor
SLC	secondary lymphoid tissue chemokine

CHAPTER ONE

INTRODUCTION

Secondary lymphoid tissues (spleen, lymph nodes, and Peyer's patches), which are strategically located at sites where antigens are likely to be captured [1], function as a meeting place that brings together rare antigen-specific lymphocytes and antigen-presenting cells. These cells then interact to mount an immune response that removes unwanted antigens from the system [2, 3]. Although these tissues differ in many ways, they all share a common feature of highly organized lymphoid compartments that are believed to enhance the efficiency of cell movements and interactions during an immune response [1]. These lymphoid compartments involve B cell-rich, dome-shaped follicles that distribute around centrally located T cell-rich zones. The factors and molecular mechanisms that regulate the formation of B-cell follicles and T-cell areas have been poorly defined. Nevertheless, several recent studies have indicated an important role for chemokines and the cytokines TNF and lymphotoxin (LT) in the development and homeostasis of lymphoid compartments within secondary lymphoid tissues. These works have been reviewed in several articles [4-6], and are briefly summarized below.

The chemokine family

Chemokines constitute a family of chemotactic cytokines that are subdivided based on the number and spacing of NH2-terminal cysteines into C, CC, CXC, and CXXXC chemokines [7] (Table 1). The majority of chemokines, including RANTES,

Ligands	Receptors
CXC chemokines	
GRO, GCP2, MIP-2	CXCR2
IP-10, MIG, ITAC	CXCR3
SDF-1 (PBSF)	CXCR4
BLC (BCA-1)	CXCR5
CXCL16	CXCR6
CC chemokines	
MIP-1α, RANTES, MCP-3	CCR1
MCP-1, MCP-3, MCP-5	CCR2
Eotaxin, RANTES, MIP-1α	CCR3
MDC, TARC	CCR4
RANTES, MIP-1α, MIP-1β	CCR5
MIP-3α (LARC)	CCR6
SLC (6Ckine), ELC (MIP-3β)	CCR7
TCA-3 (I-309)	CCR8
TECK	CCR9
CTACK	CCR10
C chemokine	
Lymphotactin	XCR1
CX3C chemokine	
Fractalkine	CX3CR1

Table 1. Mouse chemokines and their receptors. Single common names for each chemokine are provided. For some, a common alternative name is shown in parenthesis. For more comprehensive reviews on chemokines and their new systematic nomenclature see [9].

MIP-1α, and MCP-1, are induced upon various pro-inflammatory conditions in which they preferentially attract activated or memory cells [8]. In contrast with these inducible pro-inflammatory chemokines, several new chemokines have been identified; the latter are constitutively expressed within secondary lymphoid tissues and strongly attract naive, resting lymphocytes. These lymphoid tissue chemokines include BLC/BCA-1 [10, 11], SDF-1 [12-14], SLC/6Ckine [15-18], and ELC/MIP-3β [19, 20]. BLC/BCA-1 is expressed by stromal cells within the follicles of secondary lymphoid tissues. It strongly attracts resting B cells but few other cell types [10]. SDF-1 is an efficacious attractant of mature lymphocytes [14]; in lymphoid tissues, its expression is found on stromal cells in splenic red pulp and in medullary cord regions of lymph nodes (V. N. Ngo and J. G. Cyster, unpublished data). SLC and ELC are related chemokines which strongly attract naive T cells and weakly attract B cells. SLC is expressed by high endothelial venules (HEVs) in lymph nodes and Peyer's patches, and by stromal cells in the T zone of spleen, lymph nodes, and Peyer's patches [16, 17, 21]. Human ELC is highly expressed in lymphoid tissues; lymph nodes and the appendix are as especially rich sources of it [19, 20]. Expression of ELC is also high in human thymus, whereas expression in spleen is either low [19] or negative [20]. Among various cell populations tested, monocytes activated by LPS and IFN-γ in the presence of IL-10-blocking antibodies were the only cells that expressed significant amounts of ELC [20]. The identification and

characterization of mouse ELC is described in Chapter Two of this dissertation. Chemokines signal through membrane-bound receptors that belong to the seven-transmembrane receptor family (Table 1) and that couple to pertussis toxin (PTX)-sensitive heterotrimeric Gαi proteins. Signaling through chemokine receptors has been shown to induce chemotaxis, integrin activation, respiratory burst, and transcription of cytokines [22-24].

As mentioned above, lymphoid chemokines are expressed by specialized chemokine-producing stromal cells within B and T cell areas; and they have a strong chemotactic activity to naïve lymphocytes. These properties have suggested that chemokines play an important role in the organization of lymphocytes that migrate into secondary lymphoid tissues. The first demonstration that chemokines play a role in the establishment of normal cellular compartments within secondary lymphoid tissues came from a study of mice with gene-targeted deletion of BLR1/CXCR5 [25]. BLR1-deficient mice lacked several peripheral lymph nodes and also failed to develop normal architecture of B-cell follicles [25]. More recently, a spontaneous mutant mouse strain named *plt*, for paucity of lymph node T cells, was found to lack lymphoid SLC expression and to have greatly reduced ELC expression [26]. In *plt* mice and mice that have targeted inactivation of CCR7 [27], the common receptor for SLC and ELC, there is a severe paucity of T cells and dendritic cells within T zones, while follicles continue to develop.

The cytokine TNF and lymphotoxin ligand and receptor systems

TNF and lymphotoxin-α are structurally related homotrimeric proteins. They were previously identified from activated cultured cells and were known for their cytotoxic activities against tumor cell lines [28]. TNF is a homotrimer and functions both as a membrane-bound and as a secreted protein; LTα, which lacks a membrane domain, is a secreted protein [29, 30] (Fig. 1). LTα and TNF bind and activate each of two TNF receptors, TNFR1 and TNFR2 [31]. LTα also exists in a membrane-bound heterotrimer by noncovalently associating with two copies of a structurally related type II transmembrane protein, called lymphotoxin-β [32, 33]. The membrane LTα1β2 binds and activates a distinct receptor of the TNFR family, termed the lymphotoxin β receptor (LTβR) [34] (Fig. 1). The expression patterns of TNFR1 and TNFR2 are relatively broad, whereas LTβR expression is more restricted to mesenchymal cells, including stromal cells in lymphoid tissues [35].

Studies of the independent roles of TNF, LTα and LTα1β2 using gene-targeted knockout mouse models have revealed important insights into the mechanisms of lymphoid compartmentalization. Mice deficient in TNF and TNFR1 lack follicular dendritic cells (FDCs). B cells in these mice fail to form polarized follicular clusters, and instead their distribution forms a ring at the T zone periphery [36-38]. LTα-deficient mice, which are lacking membrane LTα1β2 heterotrimers and soluble LTα3 complexes

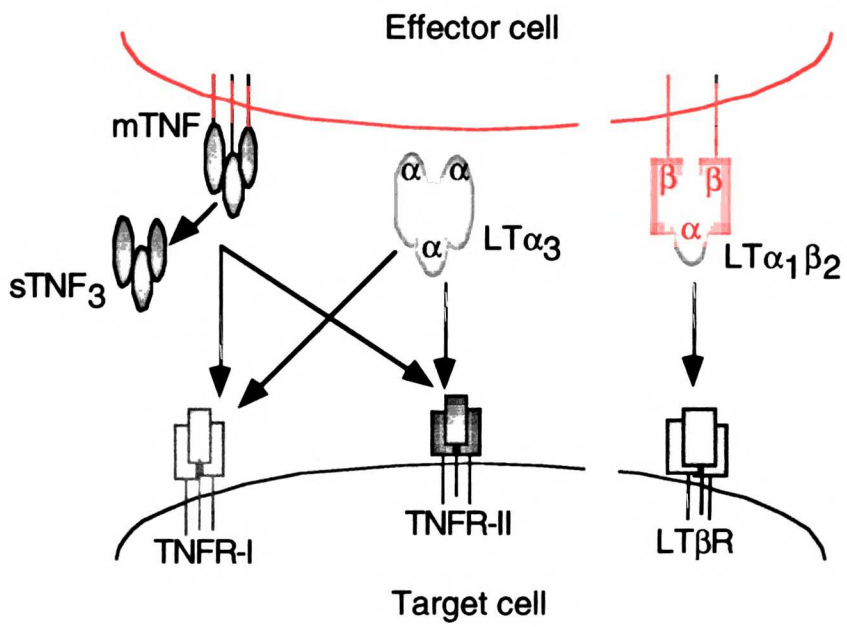

Figure 1. Schematic representations of the TNF/lymphotoxin ligand and receptor systems.

[35], show a distinct phenotype including absence of lymph nodes and Peyer's patches, and loss of splenic marginal zone cells, FDCs, and the normal B/T segregation seen in white pulp cords of the spleen [38-41]. $LT\beta^{-/-}$ mice, which cannot produce membrane forms of LT that bind LTβR but continue to express LTα, exhibit a similar phenotype except that some lymph nodes are retained and splenic architecture is somewhat less disturbed than in $LT\alpha^{-/-}$ mice [42, 43] (Korner, H., and J.D. Sedgwick, personal communications). In addition, cell transfer studies have indicated that B cells are a major source of the membrane LTα1β2, which is required for normal architecture of splenic follicles [44-46]. Together, these studies have established that the cytokines TNF, LTα and LTβ and the receptors TNFR1 and LTβR are required for normal compartmentalization of lymphocytes in the spleen.

The studies described above have established essential roles for both chemokines and the TNF/LT cytokines in the compartmental homing and development of microarchitecture within secondary lymphoid tissues. These studies have indicated that the chemokines expressed in lymphoid compartments promote homing of lymphocytes, which in turn provide TNF/LT signals to promote differentiation of the stromal cell network of secondary lymphoid tissues. However, the molecular interactions between lymphocytes and the local environment that are important in nucleating and maintaining

lymphoid compartments have remained poorly defined and will be the focus of this dissertation.

The first series of studies described in this dissertation focuses on the identification and characterization of a lymphoid tissue chemokine, which is a murine homologue of human ELC (Chapter Two). The second set of studies examines the regulation of lymphoid tissue chemokine expression and investigates a model of the interaction between BLC and LTα1β2 in lymphoid follicle development (Chapter Three). The third series of studies describes a novel role of B cells in promoting development of the splenic T zone (Chapter Four).

In summary, the studies reviewed above and those described in this dissertation have provided new insights into the development of secondary lymphoid tissue compartments. Lymphoid compartments are often ectopically induced in non-lymphoid sites in chronic inflammatory diseases, including in the joint synovium of rheumatoid arthritis, the thyroid of Hashimoto's thyroiditis patients, and the pancreas of type I diabetics. It is hoped that an understanding of the organizing mechanisms of lymphoid tissues will improve our knowledge of immunity in general and of these important human immunological diseases.

CHAPTER TWO

IDENTIFICATION AND CHARACTERIZATION OF THE CHEMOKINE ELC

ABSTRACT

Movement of T and B lymphocytes through secondary lymphoid tissues is likely to involve multiple cues that help the cells navigate to appropriate compartments. Epstein-Barr virus- induced molecule 1 (EBI-1) ligand chemokine (ELC/MIP3β) is expressed constitutively within lymphoid tissues and may act as such a guidance cue. Here, we have isolated mouse ELC and characterized its expression pattern and chemotactic properties. ELC is expressed constitutively in dendritic cells within the T cell zone of secondary lymphoid tissues. Recombinant ELC was strongly chemotactic for naïve (L-selectin^{hi}) CD4 T cells and for CD8 T cells and weakly attractive for resting B cells and memory (L-selectin^{lo}) CD4 T cells. After activation through the B cell receptor, the chemotactic response of B cells was enhanced. Like its human counterpart, murine ELC stimulated cells transfected with EBI-1/CC chemokine receptor 7 (CCR7). Our findings suggest a central role for ELC in promoting encounters between recirculating T cells and dendritic cells and in the migration of activated B cells into the T zone of secondary lymphoid tissues.

INTRODUCTION

A key function of secondary lymphoid tissues is to bring together antigen, antigen-presenting cells, and rare antigen-specific lymphocytes so that an immune response can be mounted. Antigen-bearing dendritic cells (DCs) drain into lymphoid tissues from peripheral sites of infection, migrate into the T cell-rich zone, and present antigen on surface MHC molecules [3, 47, 48]. Naive T cells enter lymphoid tissues from the blood and spend several hours migrating through the T zone, making contact with multiple DCs before returning to circulation. A successful recognition event between a T cell and an antigen-presenting DC leads to T cell retention and activation [3, 49]. Resting B cells enter lymphoid tissues through the same pathways as T cells but rapidly home into B cell-rich microenvironments, lymphoid follicles, where they reside for ~12 h before returning to circulation [50, 51]. The tropism of B cells is altered markedly upon binding antigen, with the cells relocating to the outer T zone [52, 53]. Localization of B cells in the outer T zone may play an important role in promoting encounter between antigen-specific B and T cells. The factors regulating this highly orchestrated movement of cells within the T zone and between T zone and follicles have been poorly defined, but several recent studies indicate an important role for chemokines and chemokine receptors [54].

Chemokines constitute a family of chemotactic cytokines that are subdivided based on the number and spacing of NH2-terminal cysteines into C, CC, CXC, and

CXXXC chemokines [7, 55, 56]. The majority of chemokines that have been shown to attract lymphocytes (e.g., RANTES [for regulated upon activation, normal T cell expressed and secreted], macrophage-inflammatory protein [MIP]1α, monocyte chemoattractant protein [MCP]-1) preferentially attract activated or memory cells and are unlikely to play a central role in guiding naive T or B lymphocytes through secondary lymphoid tissues [8, 57, 58]. However, over the last year, several new chemokines have been identified that are constitutively expressed within secondary lymphoid tissues and that strongly attract naive, resting lymphocytes, including secondary lymphoid tissue chemokine (SLC)/6C-kine/exodus-2/thymus-derived chemotactic agent 4 [15-18, 21], stromal cell-derived factor (SDF)1α/pre-B cell growth-stimulating factor [12-14], dendritic cell-derived CC chemokine (DCCK)1/pulmonary and activation-regulated chemokine (PARC) [59, 60], and B lymphocyte chemoattractant (BLC)/ B cell-attracting chemokine (BCA)-1 [10, 11]. SLC has been found to strongly attract naive T cells and to more weakly attract B cells. SLC is expressed by high endothelial venules (HEV) in LNs and Peyer's patches and at lower levels by other cells in the T zone of spleen, LNs, and Peyer's patches [16]. These properties suggest SLC plays a role in promoting lymphocyte passage across HEV and entry into the T cell region of lymphoid tissues. SDF1α plays a critical role in B cell and myeloid cell development [61]. SDF1α is an efficacious attractant of mature lymphocytes [14, 16] and of pre-B cells [62], and in a recent study,

has been found expressed in reticular cells located outside germinal centers in human tonsil, suggesting a role in lymphocyte trafficking to this site [63]. DCCK1 or PARC is expressed by DCs within germinal centers of human tonsil and LN that may either be follicular DCs [60] or germinal center DCs [59]. Expression in T zone DCs was reported in tonsil [59] but not LNs, and no expression was detected in spleen [60]. Since DCCK1 attracts T cells, it may play a role in promoting DC-T cell interactions [59, 60]. BLC/ BCA-1 is a recently defined chemokine expressed by stromal cells within the follicles of secondary lymphoid tissues that strongly attracts resting B cells but few other cell types [10, 11]. BLC is a ligand for Burkitt's lymphoma receptor (BLR)1, a receptor required for B cell homing to follicles in spleen and Peyer's patches [25], and this chemokine is likely to play a key role in promoting the compartmentalization of B cells into follicles [10].

ELC (also called MIP3β) is another CC chemokine that is highly expressed in lymphoid tissues. Expression analysis of human ELC has identified LNs and appendix as especially rich sources [19, 20]. Expression is also high in human thymus, whereas expression in spleen is either low [19] or negative[20]. Among various cell populations tested, monocytes activated by LPS and IFN-γ in the presence of IL-10-blocking antibodies were the only cells that expressed significant amounts of ELC [20].

The only receptor characterized to date for ELC, EBV-induced molecule 1 (EBI-1) or CC chemokine receptor (CCR)7 [19], was first identified as a molecule upregulated in EBV-transformed Burkitt's lymphoma cells [64]. The gene was later isolated from

CD4 T cells that had been infected with herpesvirus [65] and by PCR as a molecule homologous to BLR1 [66]. Northern blot analysis demonstrated that CCR7 is expressed in several B and T cell lines but not on monocytic or myeloid cell lines or cells of nonlymphoid origin. Freshly isolated PBLs show measurable CCR7 mRNA expression, and this can be upregulated by treatment with phorbol ester, PHA, or anti-CD3 [66].

The expression of ELC within secondary lymphoid tissues and the expression of its receptor CCR7 in lymphocytes suggest that ELC may function as a lymphocyte chemoattractant, perhaps promoting cell compartmentalization within these tissues. However, the only cell type that has been described to date to respond chemotactically to ELC is the T cell lymphoma line HUT78 [19]. We report here the identification of mouse ELC, and demonstrate that this CCR7 ligand is a potent chemoattractant for resting and acutely activated lymphocytes. Furthermore, we find that mouse ELC is expressed in DCs in the T cell zone of LNs, spleen, and Peyer's patches.

MATERIALS AND METHODS

Clone Identification and Sequence Analysis

BLAST searches of the National Center for Biological Information expressed sequence tag (EST) database using human ELC as a query retrieved contiguous mouse ESTs for ELC. IMAGE Consortium (LLNL) cDNA clones 1088818 and 77575 were obtained from Genome Systems, Inc. (St. Louis, MO) as EcoR1-Not1 inserts in the

pT7T3-Pac vector, and sequenced.

RNA Expression Studies

For Northern blot analysis, 10-15 μg of total RNA from mouse tissues or purified cells was subjected to gel electrophoresis, transferred to Hybond N+ membranes (Amersham Corp., Arlington Heights, IL), and probed using randomly primed ^{32}P-labeled mouse ELC EST 1088818, which spans bases 1-755 of the ELC cDNA. To control for loading and RNA integrity, membranes were stripped and reprobed with a mouse elongation factor (EF)-1α probe. For in situ hybridization (see Chapter Six for more details), frozen sections (5 μm) from C57BL/6 mice were fixed in 4% paraformaldehyde, washed in PBS, prehybridized for 1-3 h, and hybridized overnight at 60°C with sense or antisense digoxigenin-labeled riboprobes in hybridization solution. After washing at high stringency, sections were incubated with sheep antidigoxigenin antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) followed by alkaline phosphatase-coupled donkey anti-sheep antibody (Jackson Immunoresearch Labs, West Grove, PA) and developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP). Immunohistochemistry with anti-CD3, anti-B220, and anti-CD11c (PharMingen, San Diego, CA), anti-DEC205 (kindly provided by R. Steinman, Rockefeller University, NY), and MOMA-1 (kindly provided by G. Kraal, Free University, Amsterdam, The Netherlands) antibodies was as described [67]. Reverse transcription (RT)-PCR analysis of RNA from purified cell populations was with an Advantage™ RT-for-PCR kit

(Clontech, Palo Alto, CA) using a mixture of primers specific for ELC (5' primer, ggtgctaatgatgcggaagac; and 3' primer, agacacagggctccttctggt) and hypoxanthine-guanine phosphoribosyl transferase (5' primer, cctgctggattacatcaaagcactg; and 3' primer, tccaacacttcgtggggtcct).

Production of Recombinant ELC

Sequence encoding amino acids 26-108, which are predicted to encompass mature mouse ELC based on comparison with the signal peptide cleavage site of human ELC [19], was isolated by PCR and subcloned into Nde1-Xho1 sites of the pET23b vector (Novagen, Inc., Madison, WI) in-frame with the COOH-terminal HIS6-tag. TAP302 cells (kindly provided by Tracy Handel, University of California, Berkeley, CA) were transformed with the vector, the HIS-tagged chemokine was purified using NiNTA agarose (QIAGEN, Inc., Chatsworth, CA) according to the manufacturer's instructions, and elution was with 250 mM imidazole (Fisher Scientific Co., Pittsburgh, PA). Further purification was achieved using a C-18 reverse-phase HPLC column (Vydac, Hesperia, CA) and elution with an acetonitrile gradient. SDS-PAGE of this preparation showed a single band of the expected molecular mass for ELC (~9 kD) that represented >90% of the total protein. Protein concentration was measured using a protein assay (Bio-Rad Laboratories, Hercules, CA).

Cell Purification

B cells were purified by staining non-B cells with anti-CD43-biotin (PharMingen) and depletion using streptavidin-MACS® beads and a MACS® (Miltenyi Biotec, Inc., Auburn, CA). T cells were purified by staining B cells, macrophages, and granulocytes with biotinylated antibodies to B220, Mac-1, and Gran-1 (PharMingen) and depletion by MACS®, except for the RT-PCR analysis where T cells were isolated by positive selection for CD3 (Caltag Laboratories, Inc., South San Francisco, CA) by MACS®. DCs used as a source of RNA for Northern blotting were purified as described [68]. In brief, spleen or LN suspensions were prepared in RPMI containing 10% FCS, 10 mM Hepes, and antibiotics and adjusted with sodium chloride to mouse osmolarity [69] by gently pressing through a 70-μM mesh cell strainer (Fisher Scientific Co.). The suspension was layered on 2 ml of metrizamide (14.5% by weight in the same medium) solution (Accurate Chemical and Science Corp., Westbury, NY) and spun at 600 g for 10 min, and the cells at and above the interface were isolated. Flow cytometry confirmed that ~70% of the cells were CD11c+ DCs. For RT-PCR analysis, in addition to isolation from cell suspensions as above, DCs were purified from the tissue fragments that did not go into suspension by digesting in collagenase D (Boehringer Mannheim Biochemicals) at 1.6 mg/ml and bovine pancreas DNAse (Sigma Chemical Co.) at 100 μg/ml for 30 min at room temperature. EDTA was then added to a concentration of 10 mM for 5 min [70], and the digested preparation was mashed through a cell strainer. Cells were then stained with antibodies to B220, TcR, and CD11c (PharMingen) and purified to >93%

CD11c+B220- TcR- by sorting on a FACStar plus® (Becton Dickinson, San Jose, CA).

Chemotaxis

Cell suspensions were prepared from spleen, LNs, thymus, or bone marrow of C57BL/6 mice as described [53]. In the case of spleen and bone marrow, red blood cells were lysed using Tris-ammonium chloride. Chemotaxis assays were performed as described [14] using 10^6 total cells per 5-μm transwell (Corning Costar Corp., Acton, MA). Cells that migrated to the lower chamber were enumerated by collecting events for a fixed time (60 s) on a FACScan®. By counting a 1:5 dilution of input cells in the same way, the absolute number of cells that transmigrated could be determined. To determine which subsets of cells were migrating, a fraction of the cells that migrated to the lower chamber was stained and analyzed by flow cytometry. Cells from resting and activated spleen were stained with B220-FITC (clone 6B2), CD4-PE and CD8-PE (Caltag Laboratories, Inc.), and L-selectin-biotin/streptavidin cychrome (PharMingen). Migrating thymocytes were stained with CD8-FITC, CD4-PE (Caltag Laboratories, Inc.), and CD3-biotin (PharMingen), and bone marrow cells were stained with IgD^b-FITC (PharMingen), B220-PE, and IgM-biotin (Caltag Laboratories, Inc.). Granulocytes were identified by their characteristic large side scatter. For macrophage chemotaxis assays, spleen cells were first depleted of B220+, CD4+, and CD8+ cells by MACS® (Miltenyi Biotec, Inc.), and migrating cells were identified with Mac-1-PE (Caltag Laboratories, Inc.). In some experiments, cells were preactivated by incubation with phorbol-12,13-dibutyrate (PDBu,

100 ng/ml; Calbiochem Corp., San Diego, CA), 17 μg/ml polyclonal anti-mouse IgM (Jackson ImmunoResearch Labs), or 20 μg/ml LPS. Synthetic human SDF1α (N33A) synthesized by chemical ligation (a gift from M. Siani, Gryphon Sciences, South San Francisco, CA) was used as a control. This SDF1α preparation maximally attracts lymphocytes, macrophages, and granulocytes at 100-500 ng/ml [10] and therefore was used at 300 ng/ml in these studies.

Mouse EBI-1 Transfection and Calcium Fluorimetry

Mouse EBI-1 [71] was isolated by RT-PCR using a 3' primer downstream of the stop codon and a 5' primer starting at amino acid 26 of the full-length protein, truncating the hydrophobic NH_2-terminal (possible leader) peptide. Both primers contained Sal-1 sites, and the PCR product was cloned into a modified form of pREP4 (Invitrogen Corp., San Diego, CA) that contains an NH_2-terminal prolactin leader sequence and FLAG epitope followed by an in-frame Sal-1 site [72] (provided by S. Coughlin, University of California, San Francisco, San Francisco, CA). Human embryonic kidney 293 cells were transiently transfected with this construct using LipoTAXI according to the manufacturer's instructions (Stratagene Inc., La Jolla, CA). EBI-1 surface expression 2 d after transfection was confirmed by flow cytometry with anti-FLAG antibody M2 (VWR Scientific Products, West Chester, PA). Calcium mobilization studies were performed on Indo-1-loaded cells as described [73] using a fluorescence spectrometer (model 4500; Hitachi Ltd., Tokyo, Japan). Intracellular calcium concentrations were calculated using

the Hitachi 4500 intracellular cation measurement program.

RESULTS

Identification of Murine ELC Homologue

To permit the expression pattern and chemotactic activity of mouse ELC to be studied, we searched for ESTs that may encode mouse ELC using human ELC as the query sequence [19]. Seven mouse ESTs were identified in the GenBank EST database (EST nos. 1088818, 906750, 885418, 1183472, 775758, 807269, and 822508) that could be aligned into a 755-bp contig encoding a predicted protein of 108 amino acids. The high nucleotide sequence identity (84%) and amino acid identity (78%) of this sequence with human ELC (Fig. 2) strongly suggested that we had identified mouse ELC. The proteins with greatest homology to both mouse and human ELC are mouse and human SLC (Fig. 2). A major difference between ELC and SLC is the extended cysteine-containing COOH-terminal region unique to SLC. When this region is excluded from the comparison, ELC and SLC are 33% identical. The next closest match to ELC is MIP3α [20], with 23% identity. The similarity between mouse and human ELC and SLC, together with the observation that the human genes colocalize on chromosome 9 [15, 19], is consistent with the possibility that these molecules arose from a gene-duplication event, and suggests they may have related functions.

A

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   1 GCGGGCTCACTGGGGCACACACAAGCTCACTTGCACTTGGCTCCTGAACC
  51 CCTTCACGCCACAGGAGGACATCTGAGCGATTCCAGTCACTCCCCTGTGA
 101 ACCCGTCGGAGCCTCGGCCTCTCAGATTCTTGCGCACACAGTCTCTCAGG
 151 CTCACTCACTCTCTGTGGCCTGCCTCAGATTATCTGCCATGGCCCCCCGT
                                              M  A  P  R
 201 GTGACCCCACTCCTGGCCTTCAGCCTGCTGGTTCTCTGGACCTTCCCAGC
      V  T  P  L  L  A  F  S  L  L  V  L  W  T  F  P  A
 251 CCCAACTCTGGGGGGTGCTAATGATGCGGAAGACTGCTGCCTGTCTGTGA
      P  T  L  G  G  A  N  D  A  E  D  C  C  L  S  V
 301 CCCAGCGCCCCATCCCTGGGAACATCGTGAAAGCCTTCCGCTACCTTCTT
     T  Q  R  P  I  P  G  N  I  V  K  A  F  R  Y  L  L
 351 AATGAAGATGGCTGCAGGGTGCCTGCTGTTGTGTTCACCACACTAAGGGG
      N  E  D  G  C  R  V  P  A  V  V  F  T  T  L  R  G
 401 CTATCAGCTCTGTGCACCTCCAGACCAGCCCTGGGTGGATCGCATCATCC
       Y  Q  L  C  A  P  P  D  Q  P  W  V  D  R  I  I
 451 GAAGACTGAAGAAGTCTTCTGCCAAGAACAAAGGCAACAGCACCAGAAGG
     R  R  L  K  K  S  S  A  K  N  K  G  N  S  T  R  R
 501 AGCCCTGTGTCTTGAGTAAAGAGATGTGAATCACTCTGGCCCAGGAAACC
      S  P  V  S  *
 551 AAGGACCAGAAGAGAGGACCAGGCCTCCTGATGCTCTGTCCCAGACCTAA
 601 CCCAGCCAAGTCTGTGCCTAGAGAGTCGATGTGAGTGTGGACAAGAGAGT
 651 TTGTGTGGCTAGAACACCGTCTCTCTGTGGCTAGACTGCAGTGCTTCCAA
 701 TAAAGCTGCTTGGTACCGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
 751 AAAAA
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B

```
mELC   1 MAPRVTPLLAFSLLVLWTFP.APTLGGAN.DAEDCCLSVTQRPIPGNIVK
hELC   1     MAL---L-------S-.----S-T-.-----------K----Y--R
mSLC   1     MAQMMTL---S-VLALCI-WTQ-SDGGGQ----KYS-KK--YS--R
hSLC   1     MAQS--L---I-VLAFGI-RTQ-SDGG-Q----KYS--K--AKV-R

mELC  49 AFRYLLNEDGCRVPAVVFTTLR.GY.QLCAPPDQPWVDRIIRRLKKSSAK
hELC  45 N-H---IK--------------.-R.-----------E---Q--QRT---
mSLC  47 GY-KQEPSL--PI--IL-SPRKHSKPE---N-EEG--QNLM---DQPP-P
hSLC  47 SY-KQEPSL--SI--IL-LPRKRSQAE---D-KEL--QQLMQH-D-TPSP

mELC  97 NKGN.STRRSPVS..........................  (100%)
hELC  93 M-.........S..........................  (78%)
mSLC  97 G-QSPGC-KNRGTSKSGKKGKGSKGCKRTEQTQPSRG.  (33%)
hSLC  97 Q-PAQGC-KDRGASKTGKKGKGSKGCKRTERSQTPKGP  (33%)
```

Figure 2. Mouse ELC sequence and alignment with human ELC and SLC. **(A)** Nucleotide and deduced amino acid sequence of mouse ELC. The predicted signal sequence is underlined, cysteines are shown in bold, and a single potential N-linked glycosylation site is underlined. **(B)** Alignment of mouse ELC (mELC) protein sequence with those of human ELC (hELC) and mouse and human SLC (mSLC and hSLC). Identical amino acids are shown as hyphens, and dots represent gaps inserted for optimum alignment. Numbering is with respect to the first amino acid shown in the full-length protein. These sequence data are available from EMBL/ GenBank/DDBJ under accession no. AF059208.

ELC Is Expressed in T Cell Zones of Secondary Lymphoid Tissues

Northern blotting analysis revealed that mouse ELC was constitutively expressed at high levels in both mesenteric and peripheral LNs (Fig. 3). Significant expression was also identified in spleen and Peyer's patches, and low expression was seen in the thymus (Fig. 3). No expression was detected in bone marrow or in several nonlymphoid tissues (Fig. 3). To determine whether ELC was produced by a restricted set of cells within lymphoid tissues, which might suggest a role in controlling cell positioning, in situ hybridization analysis was performed (Fig. 4). Strikingly, the ELC probe hybridized strongly to a subset of cells within the T cell zone (or periarteriolar lymphatic sheath) of spleen but not to cells in the B cell follicles or the red pulp (Fig. 4 A). Within LNs, ELC-expressing cells were distributed evenly throughout the T zone (or paracortex) and were absent from follicles (Fig. 4 B). Similarly, in Peyer's patches, a hybridization signal was detected in the T cell region (the interfollicular zone) but not within follicles or in the

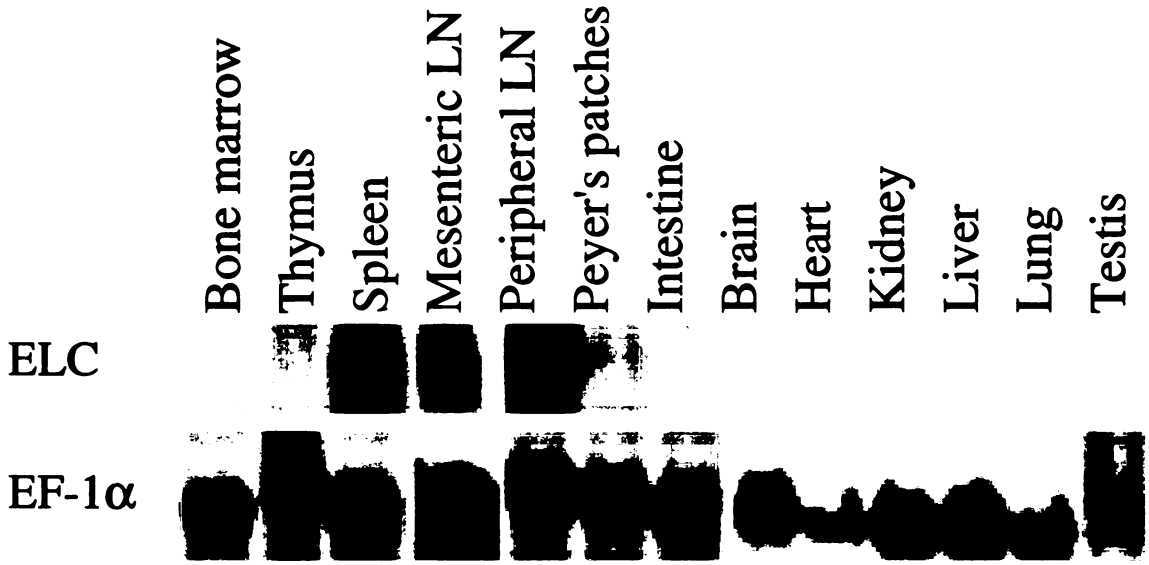

Figure 3. Northern blotting analysis of ELC expression in mouse tissues. Top, Hybridization with ELC probe. Bottom, Hybridization with EF-1α to control for amounts of RNA loaded.

subepithelial dome region (Fig. 4 C). No hybridization signal to T zones of secondary lymphoid tissues was seen with a control (sense) probe (Fig. 4 I).

ELC Is Expressed by Dendritic Cells

The distribution of ELC-expressing cells in spleen, LNs, and Peyer's patches appeared similar to the distribution of T zone DCs in these tissues [3]. To further investigate this similarity, spleen sections serial to those used for hybridization were stained to detect T cells (Fig 4. D, red) or with antibodies to CD11c [74] to detect DCs (Fig. 4 E, red). These sections were also stained with antibody to the marginal metallophilic macrophage marker, MOMA-1 [75], to outline the white pulp cords (Fig. 4, D and E, brown). The distribution of ELC-positive cells (Fig. 4 A) was very similar to the distribution of CD11c+ DCs located in the T zone (Fig. 4, A, D, and E). Interestingly, although clusters of CD11c+ DCs were also located in the bridging channels between T zone and red pulp (Fig. 4 E) as observed previously [3, 74], little ELC hybridization signal was detected in these regions (Fig. 4, A and E). Staining of a further serial section with the DC marker DEC205 that is expressed most highly on DCs in the T zone [3, 76] revealed a close concordance between the ELC hybridization pattern and the distribution of DEC205-expressing cells (Fig. 4, A and F). Combined staining for the fixation-resistant marker B220 and for ELC confirmed that the ELC-expressing cells were distributed across the T cell zone but did not extend into the B cell area of the splenic

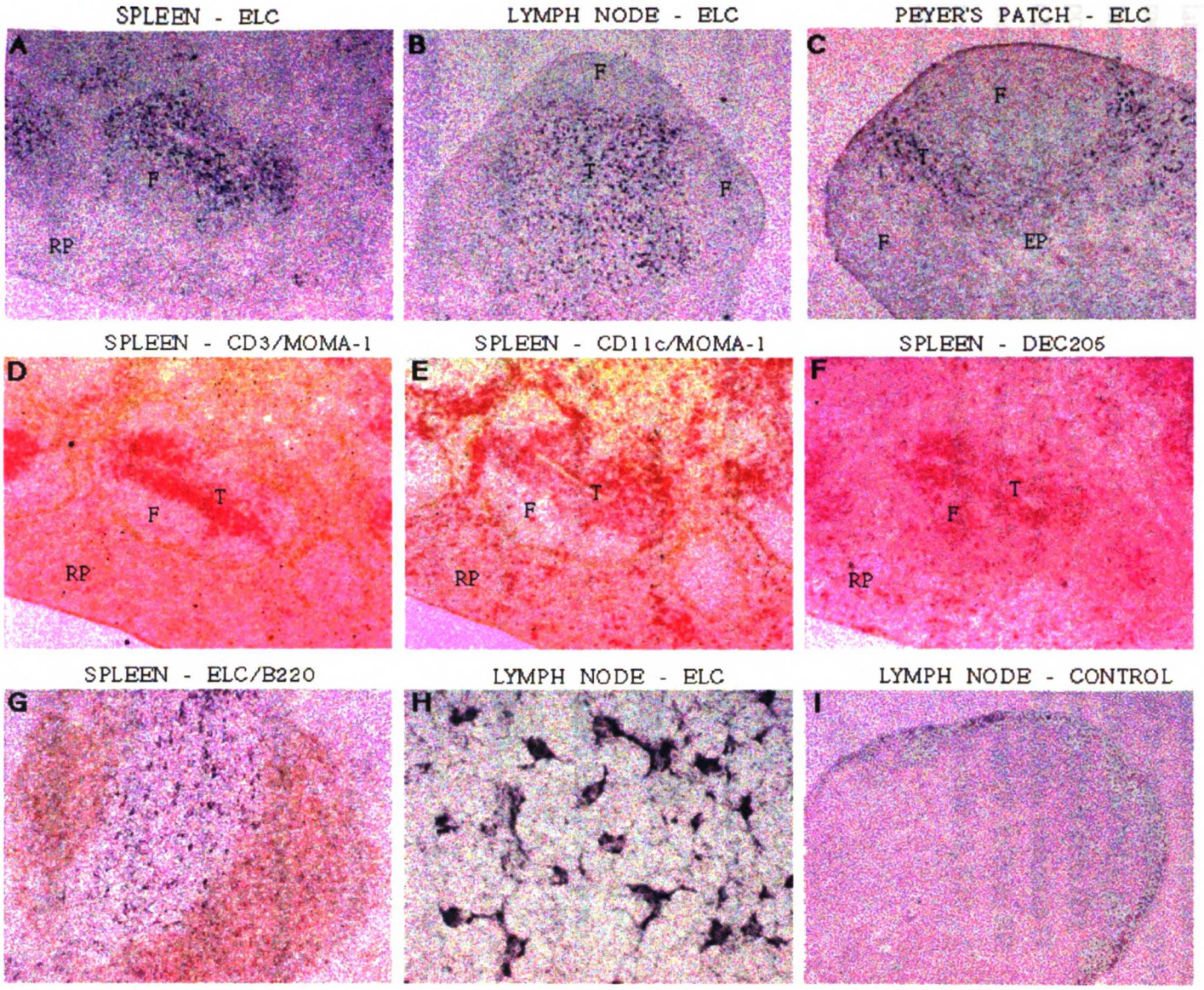

Figure 4. In situ hybridization analysis of ELC expression pattern in mouse lymphoid tissues. (A-C and G-I) Bright-field micrographs showing hybridization with digoxigenin-labeled antisense (A-C, G, and H) or sense (I) ELC probe to sections of spleen (A and G), mesenteric LN (B, H, and I), and Peyer's patch (C). Signal is seen as black staining. (D-F) Immunohistochemistry of spleen sections serial to A to detect in brown MOMA-1-positive marginal metallophilic macrophages (D and E) and in red CD3+ T cells (D), CD11c-expressing DCs (E) and DEC205-expressing DCs (F). The spleen section in G was double stained to detect B220 (brown) and ELC (black). T, T zone; F, follicle; RP, red pulp; EP, epithelium. Original magnifications: A-F and I, X5 objective; G, X10 objective; and H, X40 objective

white pulp (Fig. 4 G). Close examination of the ELC hybridization signal in the T zone showed that many of the cells had a striking dendritic morphology (Fig. 4 H).

To directly test whether DCs were a source of ELC, Northern blot analysis was performed with RNA from partially purified spleen and LN DCs (Fig. 5 A). ELC expression was detected in the DC preparations, whereas purified B cells, T cells, and macrophages did not express detectable amounts of ELC (Fig. 5 A). To rule out the possibility that the Northern blot signal derived from a small population of contaminating cells, CD11c+ splenic DCs were FACS® sorted, and ELC expression was measured by RT-PCR. In agreement with the Northern blot result, ELC expression was detected in the purified DCs but was undetectable in purified T cells, B cells, or macrophages (Fig. 5 B). It was anticipated that if DCs were the major ELC-expressing cells in the spleen, the PCR signal in the sorted DCs would be increased compared with the unsorted spleen

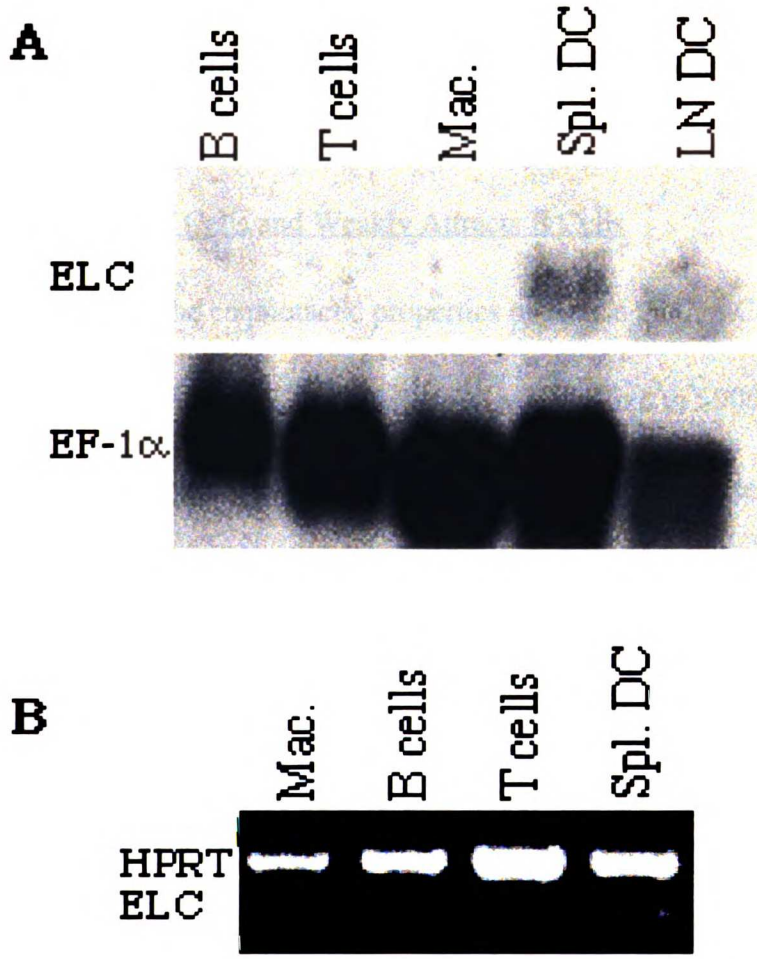

Figure 5. ELC expression in purified cells. (A) Northern blot analysis of ELC expression in MACS®-purified spleen B cells and T cells, mouse peritoneal macrophages (Mac.), and spleen (Spl.) and LN DCs purified to ~70%. EF-1α hybridization is shown to control for amounts of RNA loaded. (B) RT-PCR analysis of ELC expression in total spleen before sorting (Spl.) and in splenic CD11c+ DCs isolated by FACS® sorting (Spl. DC), and lack of expression in purified spleen B cells and T cells and peritoneal macrophages (Mac.). Primers specific for HPRT were included in each sample as a reaction control.

preparation, and this is what was observed (Fig. 5 B). Taking the findings from the in situ hybridization, Northern blot, and RT-PCR analysis together, we conclude that DCs present within the T zone are the major source of ELC in secondary lymphoid tissues.

ELC Strongly Attracts T Cells and Weakly Attracts B Cells

To characterize the chemotactic properties of mouse ELC, a COOH-terminal HIS-tagged version was expressed in *Escherichia coli* and purified to >90% by affinity- and high-pressure liquid chromatography. In migration assays with mouse spleen cells, a strong response was identified in CD4 and CD8 T cells (Fig. 6 A). Interestingly, CD4 cells were reproducibly found to be more sensitive to low ELC concentrations than CD8 cells (Fig. 6 A). B cells showed a weaker chemotactic response to ELC than both CD4 and CD8 T cells (Fig. 6 A). Strikingly, CD4 T cells of a naive (L-selectin^{hi}) phenotype showed a stronger migratory response than CD4 T cells of a postactivation or memory (L-selectin^{lo}) phenotype (Fig. 6 B). Lymphocytes from blood and LNs showed a similar response as cells from the spleen (data not shown).

Activated Lymphocytes Have an Enhanced Chemotactic Response to ELC

The ELC receptor was identified originally as a molecule upregulated in EBV-transformed B cells [64]. Other studies indicated that CCR7 is also upregulated by treatment of PBLs with phorbol ester, PHA, or anti-CD3 [65, 66]. In preliminary

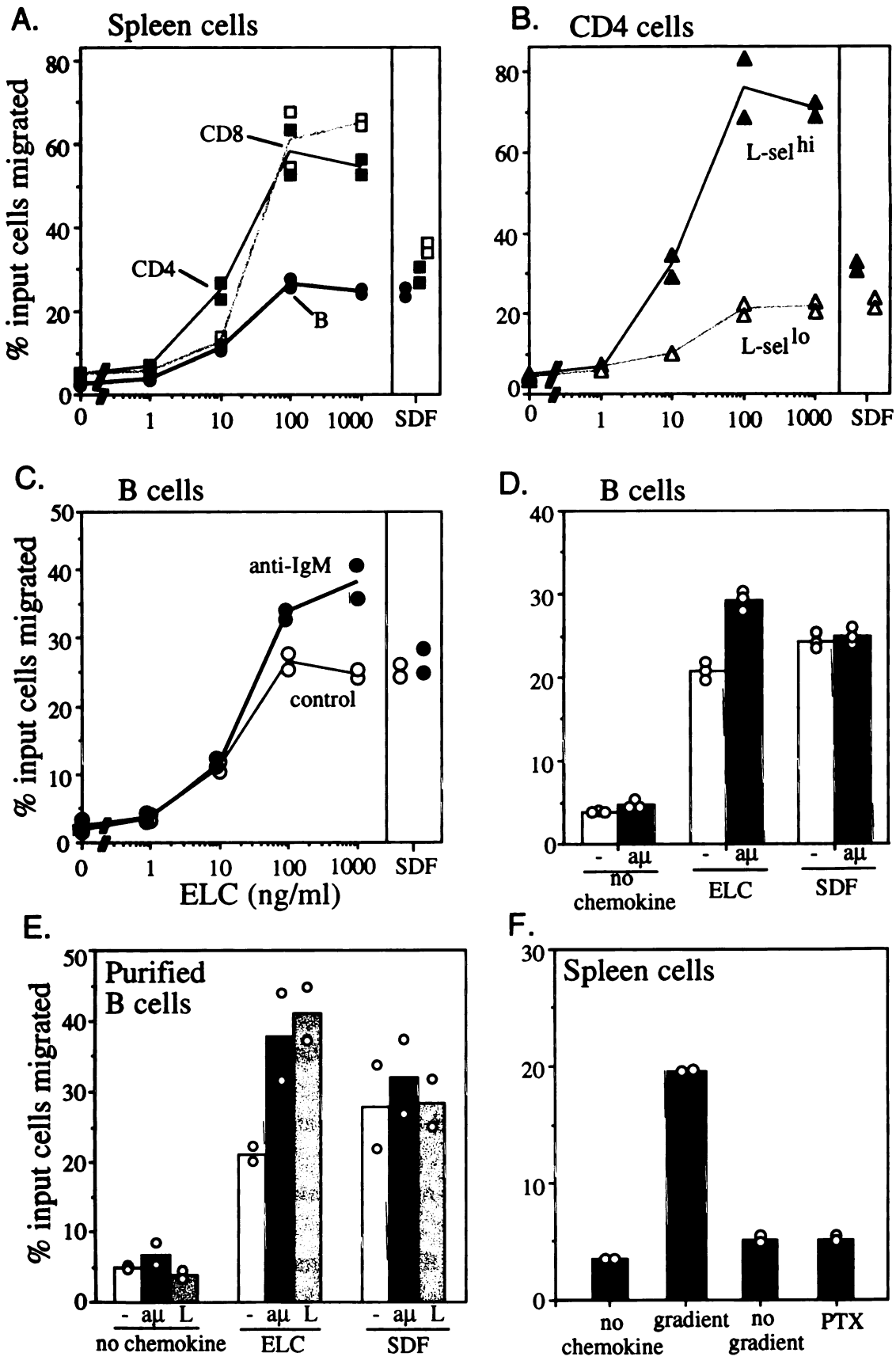
A.
Spleen cells
% input cells migrated
CD8
CD4
B
0
1
10
100
1000
SDF
B.
CD4 cells
L-sel hi
L-sel lo
C.
B cells
anti-IgM
control
ELC (ng/ml)
D.
B cells
- aμ
no chemokine
ELC
SDF
E.
Purified B cells
- aμ L
no chemokine
ELC
SDF
F.
Spleen cells
no chemokine
gradient
no gradient
PTX

Figure 6. Chemotactic activity of ELC on resting and acutely activated mouse lymphocytes. 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 spleen lymphocyte subsets: (A) CD4 T cells, CD8 T cells, and B cells; (B) L-selectin^{hi} and L-selectin^{lo} CD4 T cells; (C) B cells preincubated with anti-IgM (17 μg/ml) or media alone (control) for 4 h; (D) duplicate experiment to C with cells preincubated with media alone (-) or anti-IgM (aμ) for 4 h; (E) purified B cells preincubated with media alone (-), anti-IgM at 17 μg/ml (aμ), or LPS at 20 μg/ml (L) for 6 h; (F) total spleen cells in the absence of a gradient (no gradient, equal concentration of ELC in upper and lower chamber) or preincubated with pertussis toxin at 200 ng/ml (PTX) for 2 h. In A-E, SDF1α was included at 300 ng/ml as a positive control. In D, ELC was at 200 ng/ml, and in E and F at 300 ng/ml. The results in A-C are representative of three independent experiments, and results in D-F of two experiments.

experiments with cells cultured overnight in phorbol ester, we observed an increase in the magnitude of the B cell response to ELC (data not shown). This experiment encouraged us to test whether more physiological stimuli also increased B cell responsiveness. This was of particular interest because after engagement by antigen in vivo, B cells migrate rapidly into the outer T zone [52, 53]. Cells activated through the B cell receptor by pretreatment with anti-IgM for 4-6 h showed a 1.5-fold increase in the magnitude of their response to ELC (Fig. 6, C and D). Similar findings were made using purified B cells (Fig. 6 E), confirming that this effect was B cell intrinsic. Activation of B cells in vivo by LPS treatment also promotes migration of some cells into the T zone [77], and LPS activation was also found to enhance the B cell chemotactic response to ELC (Fig. 6 E). The increased ELC responsiveness of activated B cells did not simply reflect an overall

increase in B cell motility, as there was little difference in the frequency of activated and resting cells that migrated in the absence of chemokine (Fig. 6, C-E). Furthermore, the activated cells did not demonstrate increased responsiveness to SDF1α (Fig. 6, C-E), indicating that the enhanced response was ELC specific.

ELC Responsiveness of Thymocytes and Immature B Cells

Since ELC expression was also detected in the thymus (Fig. 3), the chemotactic response of thymocytes was measured (Fig. 7). Mature single positive thymocytes responded to ELC similarly to mature T cells in the periphery (Fig. 7 A), whereas immature ($CD4^-CD8^-$ double negative) thymocytes and most CD4/CD8 double positive thymocytes did not show a measurable response (Fig. 7 B). Interestingly, however, when the responding cells were stained with anti-CD3 antibodies, an enrichment of CD3-bright CD4/CD8 double positive cells was detected in the population that migrated in response to ELC (Fig. 7 B), indicating that the most mature fraction of double positive cells had acquired responsiveness to ELC. We also measured the ELC responsiveness of immature B lineage cells in the bone marrow. $B220^+IgM^-IgD^-$ pre-B cells and $B220^+IgM^+IgD^-$ immature B cells showed a similar dose-dependent migratory response towards ELC as mature B cells (Fig. 7 C), indicating that the cells acquire responsiveness to this chemokine early in development. Consistent with the failure to detect CCR7 expression

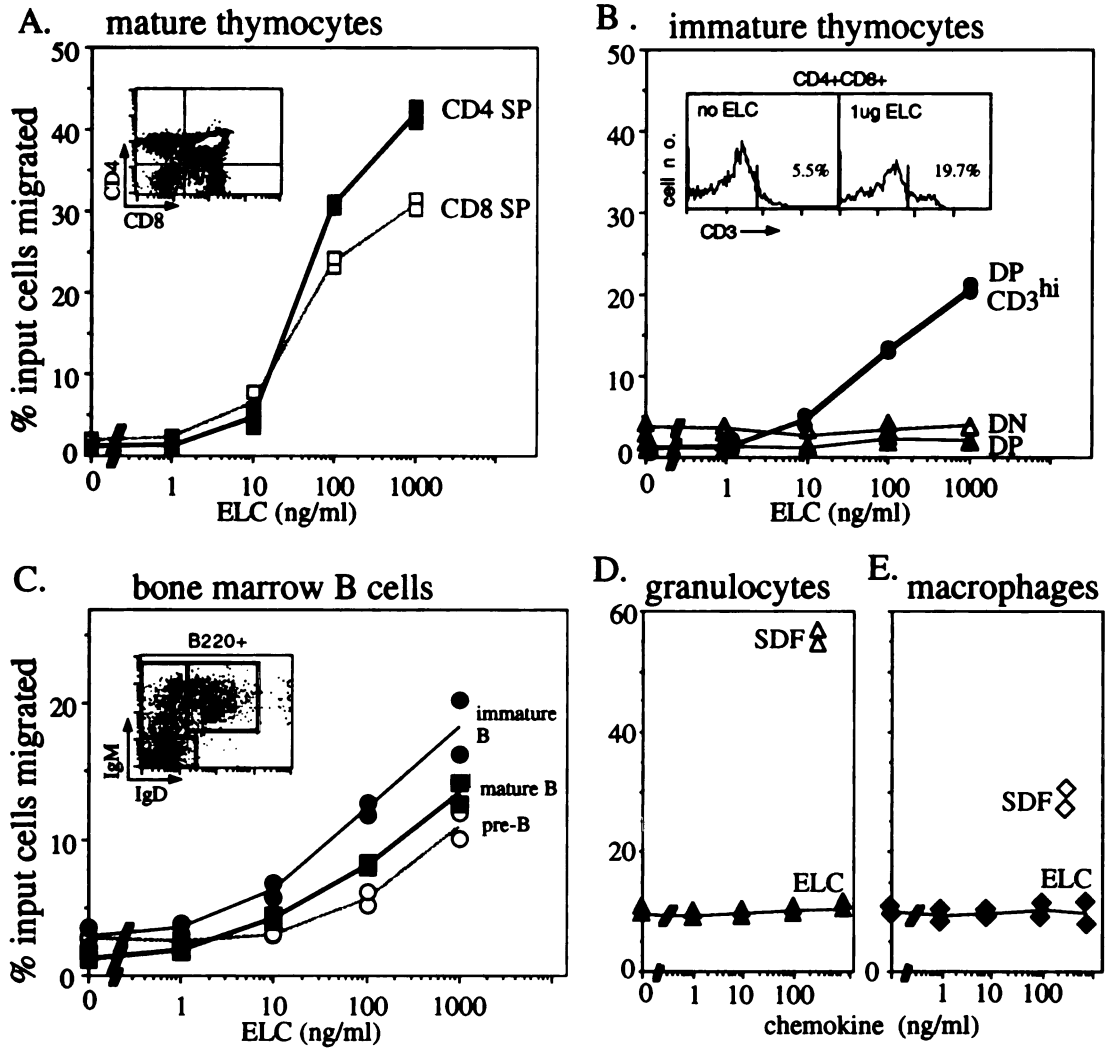

Figure 7. Chemotactic activity of ELC on immature T and B cells and lack of activity on granulocytes and macrophages. Results are expressed as the percentage of input cells of each subtype migrating to the lower chamber of a transwell filter. Panels show response of (A) mature CD4 and CD8 single positive (SP) thymocytes; (B) immature CD4/CD8 double negative (DN) thymocytes, total CD4/CD8 double positive (DP) thymocytes, and $CD3^{hi}$ double positive thymocytes; (C) bone marrow B220+IgM-IgD- cells (pre-B), B220+IgM+IgD- cells (immature B), and B220+IgM+IgD+ cells (mature B); (D) bone marrow granulocytes; and (E) spleen macrophages. Insets in A and C show flow cytometric profiles of input cells and gates used to measure the frequency of each cell type. The profile in C has already been gated for B220+ cells. The inset in B shows the gate used to identify high CD3 expression on double positive thymocytes and, as an example, the fraction of $CD3^{hi}$ double positive cells that migrated in the absence of ELC or in response to 1 μg/ml ELC. In D and E, SDF1 α (300 ng/ml) is included as a positive control. Each experiment was performed a minimum of two times.

in myeloid cells [64, 71], bone marrow granulocytes (Fig. 7 D) and spleen monocytes/macrophages (Fig. 7 E) did not respond to ELC.

Murine ELC Stimulates Murine CCR7-transfected Cells

The migration response of lymphocytes to mouse ELC was chemotactic since the cells failed to migrate when incubated in ELC in the absence of a gradient (Fig. 6 F), and the pertussis toxin sensitivity of the response (Fig. 6 F) indicated the expected involvement of a Gi-coupled receptor. To test whether the mouse homologue of human ELC we had identified was a ligand for mouse CCR7/EBI-1, 293 cells were transiently transfected with a FLAG epitope-tagged form of the receptor and assayed for mouse ELC responsiveness. CCR7-expressing cells (Fig. 8 A) showed a rapid rise in intracellular calcium in response to ELC but not to two other CC chemokines, MIP1α and MCP1 (Fig 8 B). The response of the transfected cells was dose sensitive (Fig. 8 C), and exposure to high dose ELC fully desensitized the cells to further ELC exposure (Fig. 8 B). ELC did not stimulate a calcium flux in 293 cells transiently transfected with BLR1, showing that the response of CCR7-transfected cells to ELC was specific (Fig. 8 D). These results establish that mouse ELC can stimulate cells through mouse CCR7/EBI-1.

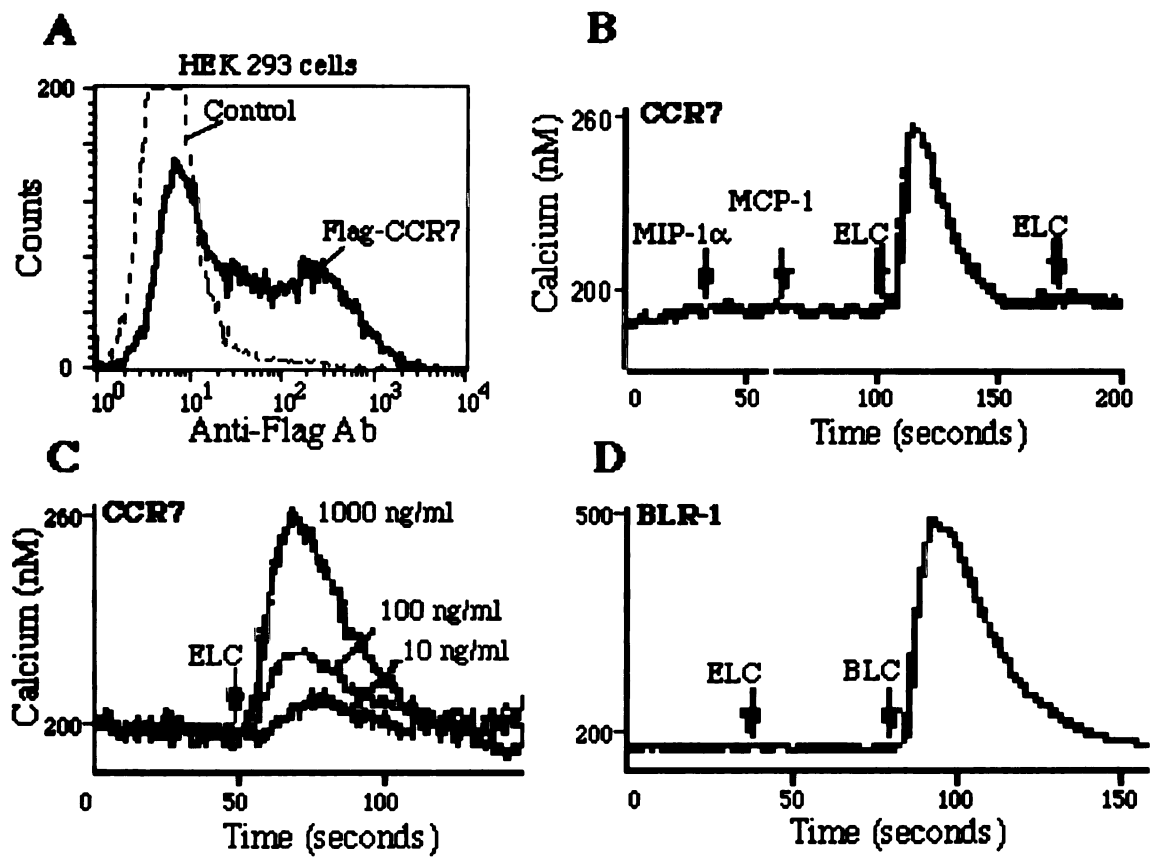

Figure 8. Mouse CCR7/EBI-1 mediated calcium mobilization in response to ELC. (A) Flow cytometric analysis of FLAG-tagged CCR7 expression on HEK293 cells transiently transfected with CCR7 expression vector (Flag-CCR7) compared with cells stained without primary antibody (Control). (B) Calcium flux of CCR7-expressing cells in response to mouse ELC (1 μg/ml) but not MIP1α (0.2 μg/ml) or MCP1 (0.2 μg/ml). (C) Calcium flux of CCR7-expressing cells as a function of ELC concentration. (D) Lack of response of BLR1-transfected HEK293 cells to ELC (1 μg/ml). BLC (2 μg/ml) was used as positive control.

DISCUSSION

In this report, we have identified mouse ELC and demonstrated that this CCR7 ligand is expressed by interdigitating DCs in the T zone of secondary lymphoid tissues. In a recent finding by others, T zone stromal cells also make ELC [78]. ELC strongly attracts resting T cells and more weakly attracts B cells. However, acute activation increases B cell responsiveness to ELC. These findings suggest two major roles for ELC: (a) to attract naive T cells into the T zone of lymphoid tissues and promote T cell-DC encounters, and (b) to attract antigen-binding B cells into the T zone of lymphoid tissues.

T and B lymphocytes enter LNs and Peyer's patches by crossing HEV located predominantly in the T zone [51, 79]. Lymphocyte attachment to HEV involves chemokine-induced activation of integrin-mediated adhesion [80]. The physiologically relevant chemokine on HEV that mediates integrin activation has not been defined, but recent studies demonstrated that SLC, SDF, and ELC could each promote integrin-mediated arrest of human lymphocytes in in vitro rolling chambers [16, 81]. The high expression of SLC by HEV cells [16] together with our failure to detect ELC expression by HEV (Fig. 4) suggests that SLC may play the more prominent role in triggering lymphocyte adhesion to HEV. Whether SDF is expressed by HEV has not yet been well characterized, and its importance at this site remains to be established. Once lymphocytes have attached to HEV, they then migrate rapidly into the T zone. Our finding that ELC is highly expressed by T zone DCs and that ELC attracts naive lymphocytes suggests that

this chemokine plays an important role in attracting cells away from HEV into the T zone and into contact with DCs. Interestingly, ELC and SLC have a strikingly similar chemotaxis profile, since both molecules attract naive T cells more efficiently than memory cells, and attract B cells more weakly than T cells. In this regard, it is interesting to note that SLC is the closest homologue of ELC (Fig. 2) and that both chemokines colocalize on chromosome 9 [15, 19], whereas most other CC chemokines cluster on chromosome 17 [55]. We are currently investigating the possibility that SLC is also a ligand for CCR7/EBI-1. Since SLC is expressed at highest levels by HEV and at lower levels by stromal cells in the T zone [16], this chemokine may be inefficient at attracting cells into the T zone once they have crossed HEV. The existence of DC-derived ELC gradients may facilitate their movement away from the HEV and in amongst the T zone DCs. A further chemokine, PARC/ DCCK1, that is expressed by DCs in germinal centers and possibly also T zone DCs, and that can attract naive T cells [59, 60], may work with ELC to promote naive T cell-DC encounters. However, in contrast to the concentrated expression of ELC in secondary lymphoid tissues in humans [19] and mice (Figs. 3 and 4), PARC/DCCK1 is expressed at highest levels in the lung [60], suggesting distinct roles for the two molecules.

Lymphocytes enter the spleen by a different route to LNs or Peyer's patches, being released in the marginal sinus that surrounds the white pulp, or at terminal arterioles in the red pulp, and then migrating into the T zone [50, 82]. However, as with

entry into LNs, entry into the lymphoid region of the spleen is pertussis toxin sensitive [83, 84], implicating the involvement of chemokines. The expression of ELC in DCs within the splenic T zone indicates that ELC is likely to play a similar role in spleen and LNs, attracting lymphocytes into the T zone and promoting T cell encounter with DCs. Interestingly, despite the absence of HEV in the spleen, SLC expression is also detected in stromal cells in the splenic T zone [16], suggesting that these two chemokines may again act in concert to attract cells into the T zone.

The responsiveness of single positive thymocytes to ELC is likely to be important in ensuring that when these cells leave the thymus, they can enter into the T zone of secondary lymphoid tissues and interact with DCs. Whether ELC plays a role in thymocyte movement within the thymus is less clear. The expression level of ELC in mouse thymus was low, and we were unable to obtain a reproducible in situ hybridization signal (data not shown). However, the acquisition of ELC responsiveness by mouse thymocytes at the $CD3^{hi}$, CD4/CD8 double positive stage suggests ELC may have a role in cell positioning within the mouse thymus. In this regard, ELC may function with several other chemokines identified recently in this tissue [21, 85-89], to help compartmentalize the cells appropriately for each developmental/selection event. Interestingly, ELC appears to be expressed at much higher levels in human thymus [19, 20], suggesting a more prominent role for ELC in human T cell development.

The weak but significant responsiveness of resting B cells to ELC is consistent with the original finding of EBI-1/CCR7 expression in B lymphocytes [64]. Previous studies have shown SLC also weakly attracts resting B cells [16], and it seems likely that ELC and SLC work together to promote migration of B cells into the T zone after they have crossed HEV or entered the marginal sinus of the spleen. Once in the T zone, the B cells may then enter the gradient of BLC that attracts the cells into follicles [10]. Interestingly, immature B cells which do not express BLR1 [90, 91] are able to migrate into the T zone of the spleen but fail to enter follicles [92, 93]. Positioning within the T zone may play an important role in the maturation or selection of these cells [93]. Since immature B cells in the bone marrow are responsive to ELC (Fig. 7 C), it is reasonable to propose that once these cells leave the marrow and enter peripheral lymphoid tissues (especially the spleen), they are able to migrate into the T zone along a gradient of ELC.

Upon binding antigen, the migration of mature recirculating B cells is altered dramatically. Rather than moving into follicles, the cells accumulate within 6-8 h in the outer T zone [52, 67]. This repositioning is likely to be critical for antigen-specific B and T cells to find each other. One mechanism by which antigen-engaged B cells might relocate to the outer T zone is through increased responsiveness to T zone-expressed chemokines. Our finding that stimulating cells through the antigen receptor increased the B cell response to ELC raises the possibility that ELC may participate in recruiting antigen-engaged B cells to this site.

In summary, our findings suggest a central role for ELC in secondary lymphoid tissue function. The high expression by T zone DCs and the strong chemotactic activity for naive T cells suggest that ELC attracts T cells into the T zone and promotes T cell-DC encounters. The weaker responsiveness of both immature and mature B cells supports a role for ELC in attracting these cells into the T zone as the necessary first step before they migrate into follicles, whereas the enhanced response of activated B cells implicates ELC as an important cue that promotes localization of antigen-engaged cells in the T cell zone. Understanding the factors that regulate ELC expression by DCs and the signals that control ELC responsiveness of lymphocytes is likely to teach us much about how secondary lymphoid tissues function in immunity.

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CHAPTER THREE

A POSITIVE FEEDBACK LOOP BETWEEN BLC AND LYMPHOTOXIN ORGANIZES LYMPHOID FOLLICLES

ABSTRACT

Mice deficient in the cytokines tumor necrosis factor (TNF) or lymphotoxin (LT) α/β lack polarized B cell follicles in the spleen. Deficiency in CXC chemokine receptor 5 (CXCR5), a receptor for B lymphocyte chemoattractant (BLC), also causes loss of splenic follicles. Here we report that BLC expression by follicular stromal cells is defective in TNF-, TNF receptor 1 (TNFR1)-, LTα- and LTβ-deficient mice. Treatment of adult mice with antagonists of LTα1β2 also leads to decreased BLC expression. These findings indicate that LTα1β2 and TNF have a role upstream of BLC/CXCR5 in the process of follicle formation. In addition to disrupted follicles, LT-deficient animals have disorganized T zones. Expression of the T cell attractant, secondary lymphoid tissue chemokine (SLC), by T zone stromal cells is found to be markedly depressed in LTα-, and LTβ-deficient mice. Expression of the SLC-related chemokine, Epstein Barr virus-induced molecule 1 ligand chemokine (ELC), is also reduced. Exploring the basis for the reduced SLC expression led to identification of further disruptions in T zone stromal cells. Finally, we show that BLC induces B cells to upregulate LTα1β2, establishing a

positive feedback loop between LTα1β2 and BLC. Together these findings indicate that LTα1β2 and TNF are required for the development and function of B and T zone stromal cells that make chemokines necessary for lymphocyte compartmentalization in the spleen. Furthermore, follicle formation and homeostasis is likely to be dependent on a positive feedback mechanism between LTα1β2 and BLC.

INTRODUCTION

Genetic studies in mice have established that the cytokines TNF, lymphotoxin α (LTα), and LTβ, and the receptors TNFR1 and LTβR, are required for normal compartmentalization of lymphocytes in the spleen [94, 95]. In TNF- and TNFR1-deficient mice, follicular dendritic cells (FDCs) are lacking and B cells fail to form follicular clusters, instead appearing in a ring at the T zone periphery [25, 36-38]. Mice deficient in LTα, which are deficient in membrane LTα1β2 heterotrimers and soluble LTα3 complexes [35], show a distinct phenotype that includes absence of lymph nodes and Peyer's patches, and loss of marginal zone cells, FDCs, and normal B/T segregation in white pulp cords of the spleen [38-41]. $LT\beta^{-/-}$ mice, which cannot produce membrane forms of LT that bind LTβR but continue to express LTα, exhibit a similar phenotype except that some lymph nodes are retained and splenic architecture is somewhat less disturbed than in $LT\alpha^{-/-}$ mice [42, 43].

In parallel studies, an understanding has begun to develop of the role played by chemokine receptors and chemokines in controlling cell movements within lymphoid tissues. Mice lacking CXCR5 (formerly Burkitt's lymphoma receptor 1 [BLR1]), a chemokine receptor expressed by mature B cells [90, 91], lack polarized follicles in the spleen and B cells appear as a ring at the boundary of the T zone [25]. Recently, a CXCR5 ligand, termed B lymphocyte chemoattractant (BLC) or B cell attracting chemokine (BCA)-1 [10, 11], has been found constitutively expressed by stromal cells in lymphoid follicles and has been proposed to act as a B cell homing chemokine [10]. Three other chemokines have been identified that are constitutively expressed in lymphoid tissues and that are efficacious attractants of resting lymphocytes: stromal cell-derived factor 1 (SDF1 [12-14]), secondary lymphoid tissue chemokine (SLC)/6Ckine [15-18, 21], and EBV-induced molecule 1 ligand chemokine (ELC)/macrophage inflammatory protein (MIP)-3β [19, 20, 96] (also see Chapter Two). SDF1 is an efficacious attractant of mature lymphocytes [14], although its pattern of expression in lymphoid tissues is not well characterized. SLC and ELC are related chemokines that strongly attract naive T cells and more weakly attract B cells. SLC is expressed by high endothelial venules (HEVs) in lymph nodes and Peyer's patches and by stromal cells in the T zone of spleen, lymph nodes, and Peyer's patches [16, 17, 21], whereas ELC is expressed by T zone DCs (see Chapter Two). The strong chemotactic activity of SLC and ELC combined with their compartmentalized expression pattern has led to the suggestion

that these molecules function in lymphocyte homing to the T zone of peripheral lymphoid tissues [16] (see Chapter Two).

The strikingly similar disruption of splenic B cell distribution in TNF- and CXCR5-deficient mice suggested that these molecules act in a common pathway to maintain follicular organization [54]. The more severe splenic disruptions in LTα/β-deficient mice suggest LT may function upstream of molecules that help organize cells into both follicles and T zones. In addition, cell transfer experiments showed that B cells are an essential source of LTα1β2 for the development of FDCs, but it was not clear whether LTα1β2 was expressed by naïve B cells [44-46]. Here we report that expression of the CXCR5 ligand, BLC, is substantially reduced in TNF-, TNFR1-, LTα-, and LTβ-deficient mice. Expression of SLC and ELC is also reduced, whereas SDF1 is unaffected. Antagonism of LTα1β2 function in the adult by treatment with soluble LTβR-Ig or anti-LTβ antibody caused reductions in BLC and SLC expression. We also observed that in addition to defects in follicular stromal cells, the LT- and TNF-deficient mice had disruptions in T zone stromal cells. To identify which cell types may act as sources of the LTα/β and TNF required for upregulating BLC expression, mice lacking subpopulations of hematopoietic cells were studied. Mice deficient in B cells, which also lack follicular stromal cells, had reduced BLC expression, whereas T cell and marginal zone macrophage (MZM)-deficient mice were unaffected. Using the soluble LTβR-Ig to stain

freshly isolated cells, we are able to detect LTβR-Ig binding on a significant fraction of total naïve B cells in wildtype spleen and lymph nodes. LTβR-Ig binding on B cells is BLC dependent as LTβR-Ig staining is significantly reduced on B cells from BLC-deficient mice. *In vitro* incubation of B cells with recombinant BLC directly induces LTα1β2 upregulation in a dose-dependent, pertussis toxin-sensitive manner. These findings suggest that TNF and LTα1β2 transmit signals required for the development and function of stromal cells that produce chemokines essential for normal organization of lymphoid tissue compartments. Mechanistically, the development and maintenance of lymphoid follicles seems dependent on the cross-regulation between LTα1β2 and BLC.

MATERIALS AND METHODS

Animals

$TNF^{-/-}$, $TNF/LT^{-/-}$, and $LT^{-/-}$ mice were generated using C57BL/6 embryonic stem (ES) cells and maintained on a pure C57BL/6 background as described [38, 97]. A further strain, generated by targeting of the LTβ gene in Bruce 4 C57BL/6 ES cells [98], was produced. The LTβ gene was disrupted by insertion of the neomycin cassette in reverse orientation in exon I, leading to complete gene inactivation and typical $LT\beta^{-/-}$ phenotype (Korner, H., D.S. Riminton, F.A. Lemckert, and J.D. Sedgwick, personal communications). $TNFR1^{-/-}$ mice were generated using C57BL/6 ES cells and were

maintained on a pure C57BL/6 background [99]. BLC$^{-/-}$ mice were generated using JM1 129 ES cells (a gift from N. Killeen) and bred onto a C57BL/6 background [100]. Most BLC$^{-/-}$ mice used in this study were mixed 129 and C57BL/6 background. C57BL/6 *op/op*, C57BL/6 BCR$^{-/-}$ (μMT), C57BL/6 TCR-β$^{-/-}$δ$^{-/-}$, and C57BL/6 recombination activating gene (RAG)-1$^{-/-}$ mice were obtained from The Jackson Labs. *op/op* mice are toothless and were fed powdered mouse chow moistened with water. Mice used for soluble LTβR-Ig [101] or anti-LTβ mAb (BB.F6 [102]) treatment were from a C57BL/6 colony maintained at the University of California San Francisco. Treatment was with 100 μg of fusion protein or 200 μg of antibody intraperitoneally once per week as described previously [103-105]. As a control for the LTβR-Ig fusion protein, which contains human IgG1 hinge, C_H2 and C_H3 regions, mice were treated with a human LFA3-IgG1 hinge, C_H2 and C_H3 region fusion protein (100 μg/wk, i.p.) as in previous studies [103, 104]. Human LFA3 does not bind to mouse CD2 [39]. The control group for the hamster anti-LTβ mAb-treated mice were injected with hamster anti-KLH mAb [105].

Northern Blot Analysis

10-15 μg of total RNA from mouse spleens was subjected to gel electrophoresis, transferred to Hybond N+ membranes (Amersham Pharmacia Biotech), and probed using randomly primed ^{32}P-labeled mouse cDNA probes of the following types: BLC, bases 10-532 [10]; SLC, bases 1-848 [16]; ELC, bases 1-755 (see Chapter Two); and SDF1α,

bases 30-370 [13]. To control for loading and RNA integrity, membranes were reprobed with a mouse elongation factor 1α (EF-1α) probe. For quantitation, Northern blots were exposed to a PhosphorImager screen for 6 h to 3 d and images were developed using a Storm860 PhosphorImager (Molecular Dynamics). Data were analyzed using ImageQuant® software (Molecular Dynamics), and chemokine mRNA levels were corrected for RNA loaded by dividing the chemokine hybridization signal by the EF-1α signal for the same sample. Relative expression levels were calculated by dividing the corrected signal for each mutant or treated sample with the mean corrected signal for the wild-type or control treated samples, as appropriate, that were included on each of the Northern blots.

In Situ Hybridization

For in situ hybridizations (see Chapter Six for more details), frozen sections (6 μm) were fixed in 4% paraformaldehyde, washed in PBS, prehybridized for 1-3 h, and hybridized overnight at 60°C with sense or antisense digoxigenin-labeled riboprobes in hybridization solution. After washing at high stringency, sections were incubated with sheep antidigoxigenin antibody (Boehringer Mannheim) followed by alkaline phosphatase-coupled donkey anti-sheep antibody (Jackson ImmunoResearch Laboratories) and developed with NBT (Bio-Rad) and BCIP (Sigma).

Immunohistochemistry

Cryostat sections (6-7 μm) were fixed in acetone and stained using the following mAbs: rat anti-B220 (RA3-6B2); rat anti-CD4 and -CD8 (Caltag); rat anti-CD35 (8C12; PharMingen); rat anti-MOMA1 (provided by Georg Kraal, Free University, Amsterdam, The Netherlands); and biotinylated mouse anti-BP-3 [106]. Rat IgG antibodies were detected with goat anti-rat-conjugated horseradish peroxidase or alkaline phosphatase (Southern Biotechnology Associates) and biotinylated antibodies with avidin-alkaline phosphatase (Sigma Chemical Co.). Enzyme reactions were developed with conventional substrates for peroxidases (diaminobenzidine/H_2O_2 [Sigma]) and alkaline phosphatase (FAST RED/Naphthol AS-MX [Sigma] or NBT/BCIP). In some cases, sections were counterstained with hematoxylin (Fisher Scientific Co.). Sections were mounted in crystal mount (Biomeda Corp.) and viewed with a Leica DMRL microscope. Images were acquired on an Optronics MDEI850 cooled CCD video camera (Optronics Engineering) and were processed with Photoshop software (Adobe Systems, Inc.).

Cell staining with the LTβR-Ig fusion protein

Spleen, lymph node, bone marrow and blood cells were prepared as described [53]. Surface expression of LTα1β2 was detected by staining cells with LTβR-Ig [101], 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 was by incubation with 10 μg/ml BBF6 [102] for 20min (see Chapter Six for more details).

Adoptive transfers.

2-4 x 10^7 spleen or blood cells from Ly5.1$^+$ donor mice were transferred by tail vein injection into congenic Ly5.2$^+$ recipients. After 0.5 or 6 hours, cells from blood, spleen, and lymph nodes were collected for flow cytometry. In some experiments, donor cells were incubated at 10^7/ml with 200ng/ml pertussis toxin (PTX; List Biological Laboratories) at 37^{0}C for 1 hour, washed, and then transferred.

Chemokines and cell culture.

Recombinant mouse chemokines were produced and used in chemotaxis assays as described [107]. 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 found on freshly isolated cells (data not shown). Therefore, B6 spleen cells (10^6 /well) were pre-incubated in flat bottom 96-well plates at 37^{0}C, 5% CO_2in 200 μl of RPMI / 10% fetal calf serum for 14-15 hours 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 (not shown).

RESULTS

Reduced Chemokine Expression in TNFR1-, TNF-, LTα-, and LTβ-deficient Mice

To explore whether TNF/TNFR1 and CXCR5 function in a common pathway of follicular organization, we measured CXCR5 expression in TNFR1- and TNF-deficient mice by flow cytometry. Splenic B cells from TNF- and TNFR1-deficient mice expressed levels of CXCR5 that were slightly elevated compared with wild-type controls ([108]; Ansel, K.M., and J.G. Cyster, data not shown). Increased CXCR5 expression seemed unlikely to account for the disrupted organization of B cells in TNF- or TNFR1-deficient animals, but could result from reduced expression of ligands that normally engage and downregulate CXCR5. Therefore, we tested whether TNF/TNFR1 regulated CXCR5 ligand expression by measuring BLC RNA levels in TNF- and TNFR1-deficient mouse spleens (Fig. 9, A and B). BLC expression was reduced approximately threefold in both types of mutant mice compared with wild-type littermates. In situ hybridization analysis of TNFR1-deficient spleen confirmed the reduced expression of BLC by follicular stromal cells (Fig. 9 C). Animals deficient in LTα or LTβ also lack follicles and follicular stromal cells, although the absence of MZMs and the severely disrupted B/T boundary make the splenic phenotype of these mice distinct. BLC expression was reduced even more severely in spleens from LTα- and LTβ-deficient animals than from TNF-deficient mice (Fig. 9, A and B), and the residual expression was too low to be detected in in situ

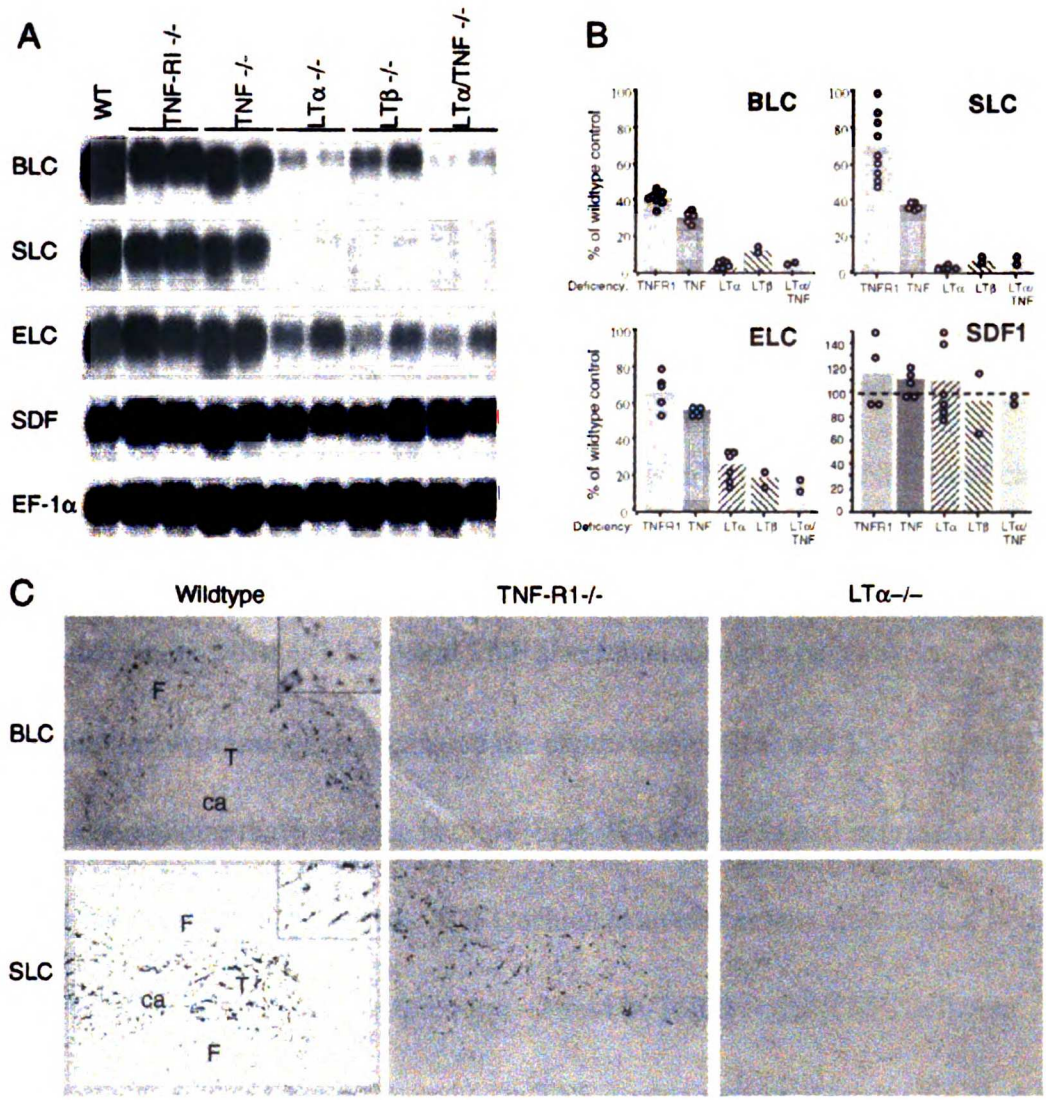

Figure 9. Reduced expression of lymphoid tissue chemokines in TNF/TNFR1- and LTα/β-deficient mouse spleen. (A) Northern blot analysis of total splenic RNA of the indicated mice to detect expression of BLC, SLC, ELC, and SDF1. Hybridization to EF-1α was used to control for RNA loaded. The hybridization signals for SDF1α and SDF1β were similar and the signal for SDF1α is shown. WT, wild-type. (B) Relative chemokine mRNA levels as determined by PhosphorImager analysis of the Northern blot shown in A and additional blots, after correcting for RNA loading from the corresponding EF-1α value. Data from individual mice are shown as open circles and means as shaded bars. (C) In situ hybridization analysis of BLC and SLC expression in spleen from wildtype, TNFR1$^{-/-}$, or LTα$^{-/-}$ mice. Original magnification: X10. ca, central arteriole; F, follicle; T, T zone. The insets in the BLC and SLC wildtype control panels are included to show the morphology of the chemokine-expressing stromal cells (original magnification: X40).

hybridization analysis (Fig. 9 C). In mice deficient in both LTα and TNF, BLC expression was reduced to an extent similar to LTα single mutants (Fig. 9, A and B), consistent with the possibility that these cytokines function in a common pathway leading to BLC expression.

Splenic T zone organization is also disrupted in the cytokine- and cytokine receptor-deficient animals, ranging from subtle changes in TNF- and TNFR1-deficient mice to almost complete loss of T zones in LTα, LTβ, and LTα/TNF double mutant mice. To determine whether LTα/β and TNF also functioned in a pathway leading to T zone chemokine expression, we measured the expression of SLC and ELC, related T cell attracting chemokines that are made in the T zone. We also measured expression of the more broadly distributed chemokine, SDF1, which is an efficacious attractant of both B and T cells. SLC was reduced in expression ~2-fold in TNFR1- and TNF- deficient animals and >20-fold in LTα, LTβ, and LTα/TNF double mutant animals (Fig. 9, A and B). By in situ hybridization analysis, the network of SLC expressing stromal cells remained visible in the TNFR1 mutant mice but could not be detected in LTα-deficient animals (Fig. 9 C). Expression of ELC was also reduced in all of the mutant strains, although less severely than SLC (Fig. 9, A and B). By contrast, SDF1 expression was not significantly reduced in any of the mutant animals (Fig. 9, A and B), indicating that the reductions in BLC, SLC, and ELC are physiologically relevant and not the result of an

overall decrease in chemokine gene expression. It should be emphasized that the TNFR1 mutant and the four cytokine mutants used in this study [38, 97, 99] (Korner, H., D.S. Riminton, F.A. Lemckert, and J.D. Sedgwick, personal communications) were all generated using C57BL/6 ES cells and maintained on a C57BL/6 background, making it unlikely that any of the differences we observed in chemokine expression are due to linked genetic differences. Therefore, these experiments demonstrate that TNF/TNFR1 and LTα/β are required for normal expression of BLC, SLC, and ELC in the spleen.

Treatment of Adult Mice with LTα/β Antagonists Diminishes Chemokine Expression

To determine whether the requirement for LTα and LTβ in the expression of BLC and SLC was developmental or constitutive, we treated adult mice for various time periods with soluble LTβR-Ig fusion protein [39, 109], an antagonist of LTα1β2, and a related molecule, LIGHT [110]. Control mice were treated for equal periods of time with an LFA3-Ig fusion protein [39]. After 1 wk of LTβR-Ig treatment, splenic BLC expression was reduced twofold compared with the controls (Fig. 10 A). A further reduction in BLC expression occurred after 2 wk of treatment and did not become more severe after 3 or 4 wk of treatment (Fig. 10 A). 2 wk of treatment also lead to decreased BLC levels in mesenteric lymph nodes (Fig. 10 B). Expression of SLC was reduced in spleen and mesenteric lymph nodes of mice given LTβR-Ig, although the degree of

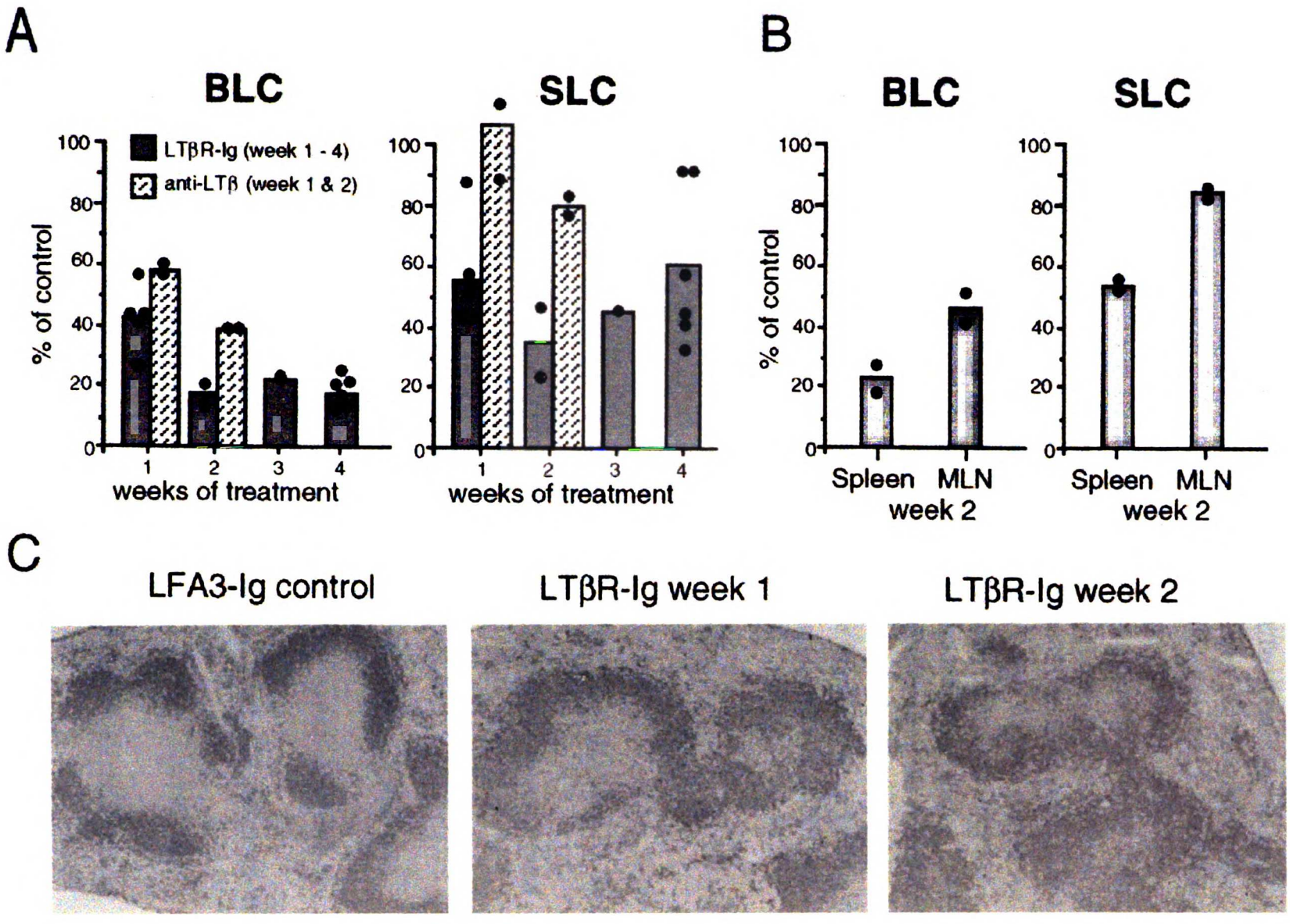

Figure 10. Decreased BLC expression in mice treated with LTα1β2 antagonists. (A) Relative chemokine mRNA levels as determined by Northern blot and PhosphorImager analysis of total spleen RNA from mice treated for the indicated time period with LTβR-Ig (100 μg/wk, i.p.) or hamster anti-LTβ mAb (200 μg/wk, i.p.). Control mice for the LTβR-Ig treatment were treated with equal doses of LFA3-Ig, and controls for the mAb treatment were given hamster anti-KLH mAb. Each sample was corrected for differences in RNA loading using the value obtained with an EF-1α probe. Chemokine expression as percentage of control was calculated by dividing the corrected value for each treated mouse with the mean corrected value for the controls at that time point. Data from individual mice are shown as filled circles and means as shaded bars. (B) Relative chemokine mRNA levels in spleen and mesenteric lymph nodes from animals treated for 2 wk with LTβR-Ig (100 μg/wk, i.p.). Calculations were made as in A. (C) Disrupted follicular organization in LTβR-Ig-treated mice. Spleen tissue from mice treated with LFA3-Ig for 2 wk or LTβR-Ig for 1 or 2 wk was sectioned and stained with B220 (dark gray) to detect B cells.

inhibition was variable and less severe than the reduction in BLC (Fig. 10, A and B). To distinguish the possible contribution of LIGHT from that of LTα1β2, mice were treated for 1 or 2 wk with an anti-LTβ mAb that specifically blocks LTα/β heterotrimer function [105, 109]. Analysis of splenic RNA showed that BLC and SLC expression were both reduced after 2 wk of anti-LTβ mAb treatment (Fig. 10 A). These results establish a key role for LTα1β2 in maintaining normal chemokine expression. Although the lesser effect of the antibody treatment compared with LTβR-Ig treatment (Fig. 10 A) suggests that LIGHT might also contribute, the results may equally be explained by the mAb causing less complete inhibition of LTα1β2 function, as has been observed in in vitro studies [109]. To explore further the relationship noted in the mutant mice between chemokine

deficiency and loss of follicular organization, spleen sections from LTβR-Ig-treated mice were stained for B cell markers as well as FDCs and marginal zone markers. As observed previously [104], expression of mucosal addressin cell adhesion molecule (MAdCAM)-1 and FDC markers were reduced after 1 wk of treatment and were undetectable by 2 wk, whereas loss of the marginal zone metallophilic macrophage (MMM) marker MOMA1 was more gradual (data not shown). A change from follicles to a ring of B cells around the T zone was also observed after 1 wk of treatment (Fig. 10 C) and was maximal after 2 wk of treatment (Fig. 10 C), paralleling the decrease in BLC expression. These results establish a constitutive requirement for LTα1β2 in maintaining normal levels of BLC and SLC and also suggest that an approximately 3-fold reduction in BLC is enough to affect the follicular organization. The more modest decrease in BLC and SLC expression in LTβR-Ig-treated mice compared with $LT\alpha^{-/-}$ or $LT\beta^{-/-}$ mice could reflect incomplete blocking of LTα1β2 function but is also consistent with a role for LTα1β2 in development that does not continue in the adult mouse.

TNF- and LTα1β2-dependent Stromal Cells in Follicles and T Zone

The above experiments demonstrated that TNF, LTα, and LTβ are required for normal expression of the chemokines BLC and SLC by stromal cells in the spleen. Several studies have established that the organization of FDCs is disrupted in TNF-,

LTα-, and LTβ-deficient mice [94], indicating that the cell type normally producing BLC might be disrupted. However, disruption of FDC organization could not account for the decreased SLC expression, since FDCs do not extend into the T zone. To test whether changes in addition to reduced SLC expression could be detected in T zone stromal cells, we examined expression of BP-3, a marker for an extensive network of stromal cells in both the T zone and follicles [106, 111]. Strikingly, the network of BP-3+ cells was greatly reduced in both the B and T zones of TNF- and TNFR1-deficient mice (Fig. 11, and data not shown) and was undetectable in LTα- and LTβ-deficient mice except for a small number of cells with altered morphology that were occasionally observed (Fig. 11). The disruption of BP-3-expressing stromal cells in both TNF- and LTα/β-deficient spleens appeared more severe than in lymphocyte-deficient (RAG-$1^{-/-}$) spleens (Fig. 11). BP-3 expression in T zone and follicles was also markedly disrupted after 1 wk of treatment with LTβR-Ig or anti-LTβ antibody and was almost undetectable after 2 wk of treatment (Fig. 11, and data not shown). This period of treatment is also sufficient to disrupt staining for MAdCAM-1 and FDC markers [104, 112]. To determine the relationship between BP-3-expressing cells in follicles and the cell types previously defined as TNF- and LTα and β-dependent, sections from wild-type mice were double stained for MAdCAM-1 or CR1 (CD35) and BP-3 (Fig. 12 A). BP-3-expressing cells in the outer follicle appeared to line the marginal sinus, and in some cases these cells

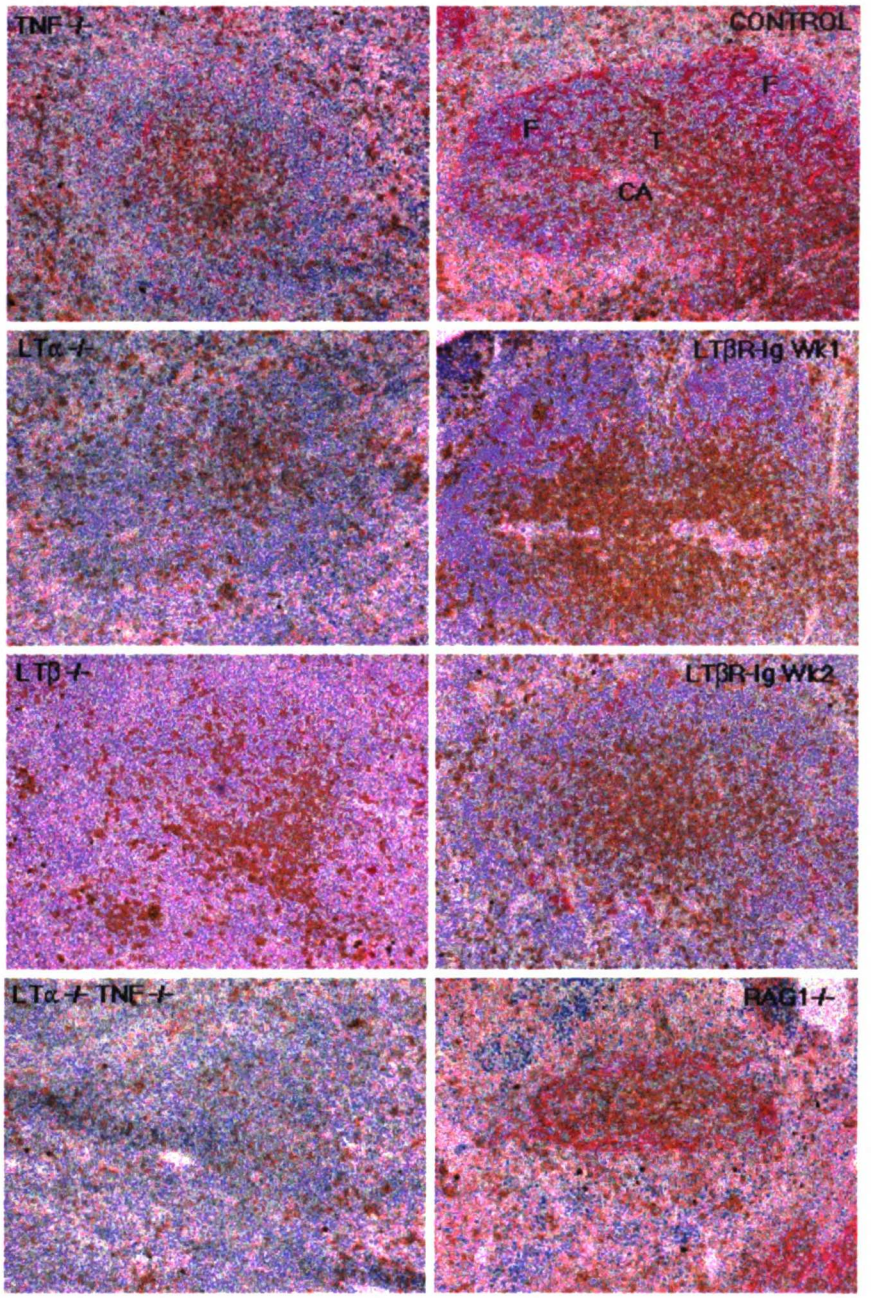

Figure 11. Disruption of BP-3 expression in follicles and T zone of TNF-, TNFR1-, LTα-, and LTβ-deficient mice and LTβR-Ig-treated mice. Spleen tissue from the indicated mutant mice or mice treated with soluble LTβR-Ig for 1-2 wk or from a wild-type control was sectioned and stained to detect T cells (combination of anti-CD4 and anti-CD8; brown) and BP-3 (red). The CD4 and CD8 staining in the RAG-$1^{-/-}$ spleen does not represent T cells, as there was no staining for CD3 (not shown). Blue staining was from counterstain with hematoxylin. CA, central arteriole; F, follicle; T, T zone. Original magnification: X10

costained for MAdCAM-1 (Fig. 12 A). Many of the BP-3$^+$ cells located in the center of the follicle costained with CD35 (Fig. 12 A), whereas BP-3$^+$ cells in other parts of the follicle, especially cells near the marginal sinus, were CD35-low or -negative (Fig. 12 A). Therefore, the BP-3- expressing stromal cell population includes T zone stromal cells (Fig. 11 and Fig. 12 A), FDCs, marginal sinus lining cells, and follicular stromal cells that are low or negative for FDC markers (Fig. 12 A). The severe disruption of BP-3 expression in the mutant mice, together with the reduced BLC and SLC expression and the loss of FDCs, indicates that TNF, LTα, and LTβ have a broad role in inducing and maintaining stromal cell integrity in T zones and B zones of lymphoid tissues.

MZMs Are Not Required for BLC Production

In addition to defects in FDCs, MAdCAM-1$^+$ cells, and BP-3$^+$ cells, LTα- and LTβ-deficient mice also lack MZMs and MMMs [42, 43, 94]. To test the possibility that the deficiency in these macrophage populations in LTα$^{-/-}$ and LTβ$^{-/-}$ mice contributed to the greatly reduced BLC expression and loss of follicular organization, we characterized spleens from *op/op* mice, a strain that is deficient in MMMs and MZMs due to a mutation in the colony stimulating factor 1 gene [113, 114]. Organization of B cell follicles appeared normal in *op/op* spleen (Fig. 12 B), and BLC expression was not reduced (Fig. 13). Expression of BP-3, MAdCAM-1, and CD35 was also not disrupted (Fig. 12 B, and

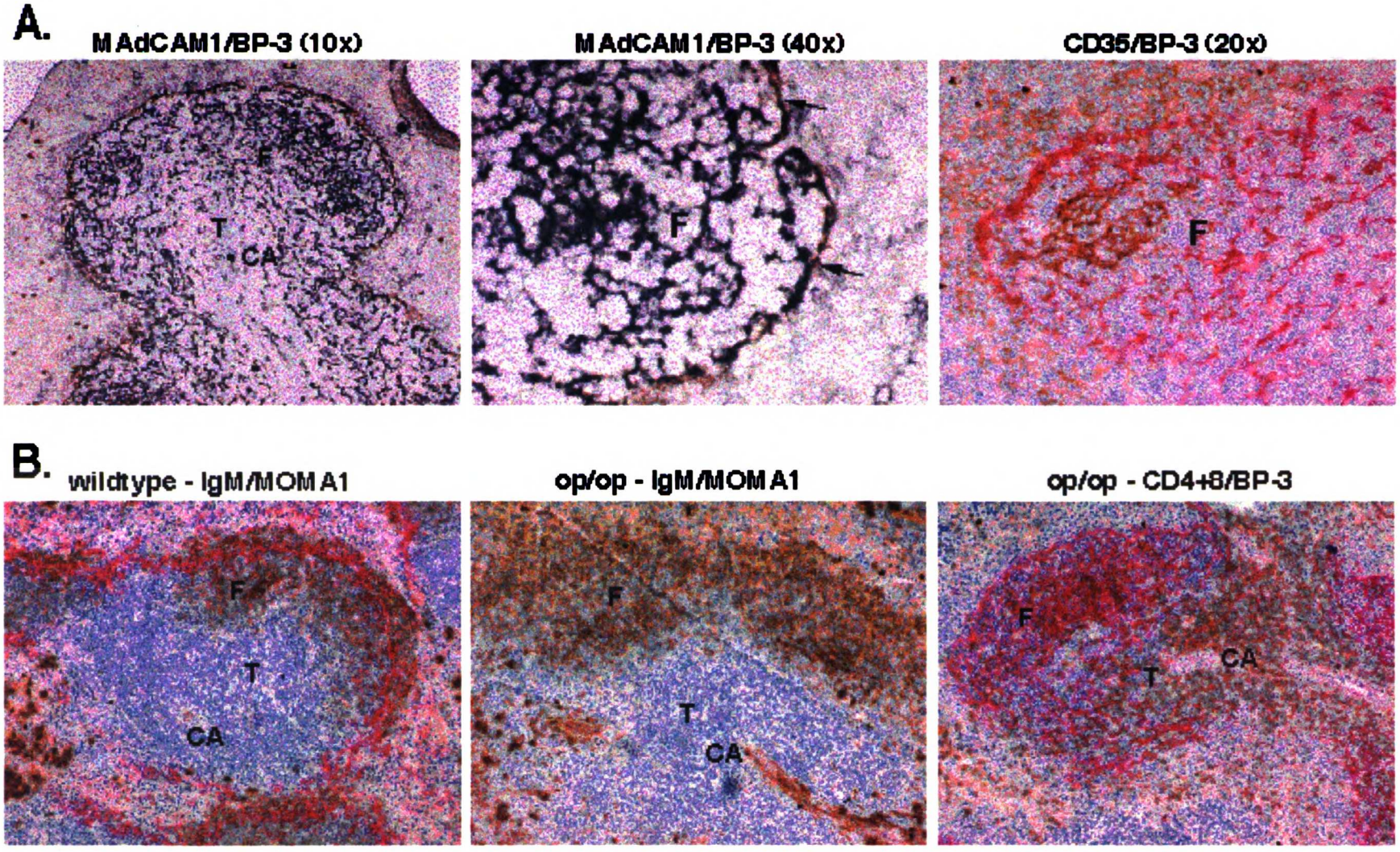

Figure 12. Costaining of BP-3+ stromal cell subsets with MAdCAM-1 and CD35 (CR1) and normal follicular organization and BP-3 expression in *op/op* mice. (A) Spleen tissue from wild-type mice was sectioned and stained to detect MAdCAM-1 (brown) and BP-3 (black; left and center panels), or CD35 (brown) and BP-3 (red; right panel). Arrows in center panel indicate MAdCAM-1 and BP-3 double-stained cells. The faint brown CD35 staining corresponds to $CD35^{high}$ marginal zone B cells and $CD35^{low}$ follicular B cells. Original magnification: X10, X20, or X40, as indicated. (B) Spleen tissue from wild-type (left) or *op/op* (center and right) mice was sectioned and stained to detect: IgM (brown) and MOMA1 (red; left and center), or CD4 and CD8 (brown) and BP-3 (red; right). Note the lack of MOMA1+ MMM staining in the *op/op* mutant. Blue staining was from counterstain with hematoxylin. Original magnification: X10. CA, central arteriole; F, follicle; T, T zone.

data not shown). These findings demonstrate that MZMs and MMMs do not make a significant contribution to the constitutive production of BLC, and also establish that these cells are not required as a source of TNF or LTα1β2 to maintain BLC expression or follicular organization.

Normal Expression of BLC Is Dependent on B Cells

Recent studies have demonstrated that B lymphocytes are an essential source of membrane LTα1β2 for establishing FDC networks and follicular organization [44, 45]. However, mice congenitally deficient in LTα have a more severe disruption of lymphoid compartmentalization than mice lacking only in lymphocyte LTα expression, indicating that there is also a nonlymphocyte source of LTα [45, 115]. To determine whether either

or both sources of LTα were required for induction of BLC, chemokine expression levels in RAG-1$^{-/-}$, B cell receptor (BCR)$^{-/-}$, and TCR$^{-/-}$ mice were compared with levels in LTα$^{-/-}$ animals. BLC expression was reduced approximately fivefold in spleens from lymphocyte-deficient (RAG-1$^{-/-}$) and B cell-deficient (μMT) mice, but were not reduced in T cell-deficient (TCR-β$^{-/-}$δ$^{-/-}$) mice (Fig. 13), demonstrating that B cells are important for induction of BLC expression, presumably providing LTα1β2 and possibly also TNF. However, BLC levels in RAG-1$^{-/-}$ and BCR$^{-/-}$ mice were not reduced to the extent of LTα$^{-/-}$ or LTβ$^{-/-}$ mice (Fig. 13, also see Fig. 9), indicating that some BLC expression in the spleen is induced by LTα/β-expressing cells other than B and T lymphocytes. Alternatively, It is possible that in the absence of lymphocytes in RAG-1$^{-/-}$ and BCR$^{-/-}$, the BLC mRNA produced by stromal cells was enriched in the total RNA pool. Treating BCR$^{-/-}$ mice with LTβR-Ig is therefore needed to determine whether a non-lymphocyte source of LTα1β2 exists.

Resting B cells express membrane LTα1β2

As mentioned above, cell transfer experiments have shown that B cells are an essential source of LTα1β2 for development of FDCs, but it is not yet clear what regulates LTα1β2 expression by naïve B cells [44-46]. Although B cells express LTβ constitutively [33, 116, 117], expression of LTα has only been reported following

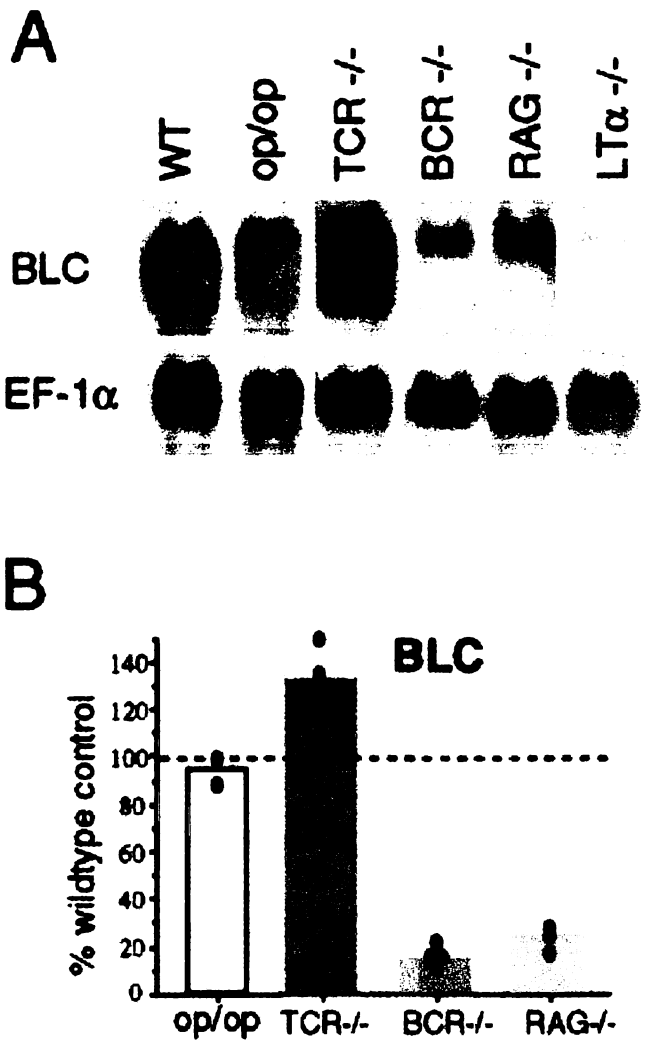

Figure 13. MZM independence and B lymphocyte dependence of BLC expression. (A) Northern blot analysis of total RNA isolated from spleen tissue of *op/op*, TCR-$\alpha^{-/-}\beta^{-/-}$ ($TCR^{-/-}$), μMT ($BCR^{-/-}$), and RAG-$1^{-/-}$ mice, probed to detect expression of BLC and EF-1α. (B) Relative chemokine mRNA levels as determined by PhosphorImager analysis of the Northern blot shown in A and additional blots, after correcting for differences in RNA loading from the corresponding EF-1α value.

exposure to activating stimuli such as CD40L or interleukin (IL)-4 [116] or treatment with endotoxin or phorbol esters [102]. However, development of primary B cell follicles is not associated with B cell activation, as it occurs in germ-free mice [118], immunoglobulin (Ig)-transgenic mice [119], T cell deficient mice [120], CD40-deficient mice [121] and IL4-deficient mice [122]. To investigate the possibility that LTα1β2 expression on naïve B cells might be downstream of BLC-mediated recruitment, we first asked whether it was possible to detect surface LTα1β2 on freshly isolated B cells (Fig. 14 a-g). A significant fraction of total B cells in wildtype spleen showed low but detectable LTβR-Ig binding compared to LTα- or LTβ- deficient spleen cells (Fig. 14 c, d, g). An even larger fraction of LN B cells stained with LTβR-Ig (Fig. 14 e, f, g) whereas B cells in the blood were mostly negative (Fig. 14 a, b, g). Similar levels of LTα1β2 were observed on B cells in anti-hen egg lysozyme (HEL) Ig-transgenic animals (Fig. 14 g), demonstrating that the expression was not induced by antigen.

LTα1β2 expression by B cells is BLC-dependent and pertussis toxin-sensitive

The findings that peripheral blood B cells express less LTα1β2 (compared to spleen and LN B cells [Fig. 14 a, c, e]) have led us to determine whether B cell expression of LTα1β2 is dependent on the presence of BLC. We then tested LTα1β2 expression on B cells in BLC-deficient animals (Fig. 15 a). While LTα1β2 expression

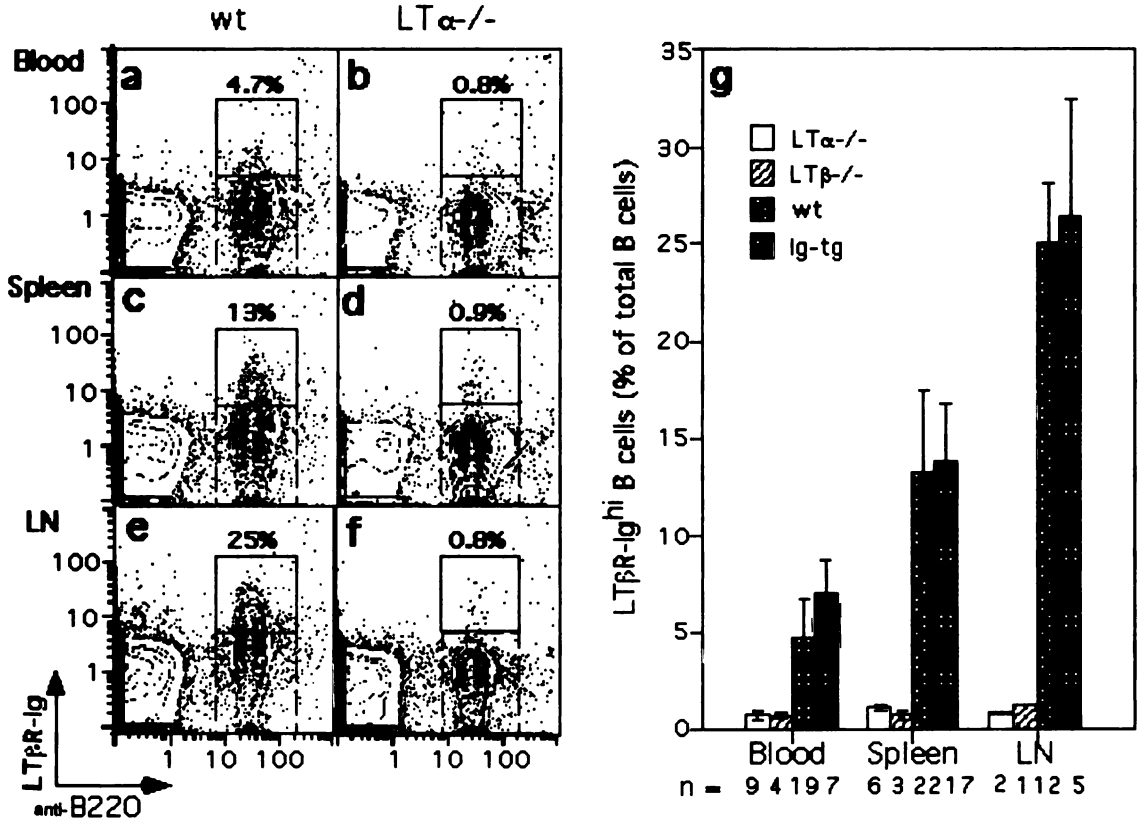

Figure 14. Membrane LTα1β2 expression on B cells in peripheral lymphoid tissues. **a-f,** Flow cytometric analysis of cells from wildtype (**a**, **c**, **e**) and LTα$^{-/-}$ (**b**, **d**, **f**) blood (**a**, **b**), spleen (**c**, **d**), and LNs (**e**, **f**) stained for LTβR-Ig and B220. In **f**, LN cells were from irradiated wildtype mice that had been reconstituted for 8 weeks with LTα$^{-/-}$ 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 LTβ$^{-/-}$ and HEL-specific Ig-transgenic mice. LTα$^{-/-}$ or LTβ$^{-/-}$ mice serve as specificity controls for LTβR-Ig staining.

on $BLC^{-/-}$ peripheral blood B cells was not different from wildtype controls, expression on B cells from spleen and LNs was significantly reduced (Fig. 15 a), 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 hours after transfer, recipient splenocytes were harvested and stained with anti-Ly5.1 to detect the transferred cells, and with LTβR-Ig (Fig. 15 b). 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. 15 b). However, six hours post-transfer, when many of the transferred cells are in BLC^+ follicular areas (not shown), LTα1β2 expression was readily detectable (Fig. 15 b). 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 15 b). 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 hours of transfer, untreated spleen B cells

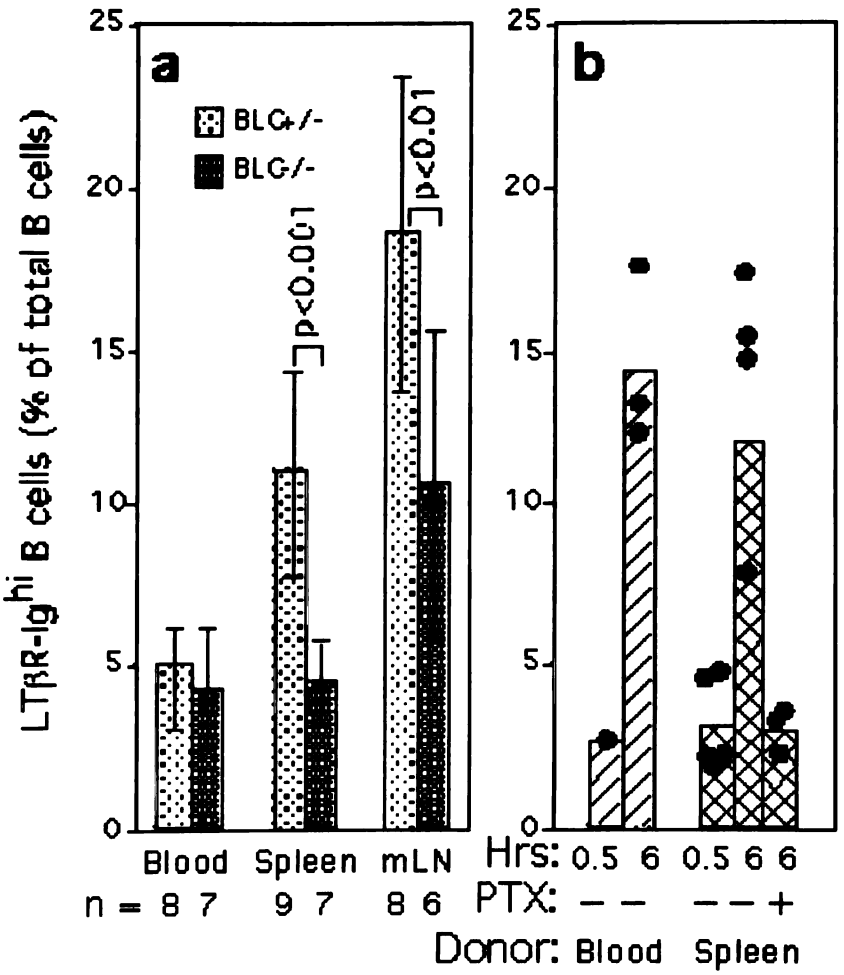

Figure 15. LTα1β2 expression on B cells is BLC dependent and PTX sensitive. **a,** LTα1β2 expression levels on B cells from blood, spleen and mesenteric LNs (MLN) of $BLC^{-/-}$ animals compared with B cells of $BLC^{+/-}$ littermate controls. Cells were gated as in (**Fig. 14 a-f**). Bars represent 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. **b,** 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.

expressed amounts of LTα1β2 equal to endogenous B cells (Fig. 15 b). 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. 15 b). Furthermore, minimal LTα1β2 upregulation occurred when B cells from wild-type mice were transferred to BLC-deficient recipients (Fig. 16 a, b). Interestingly, when B cells from BLC-deficient donors were transferred to wildtype recipients, an exaggerated induction of LTα1β2 occurred (Fig. 16 d), 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.

Recombinant BLC directly induces LTα1β2 upregulation on B cells *in vitro*

To test whether BLC functions directly to induce LTα1β2 expression, we incubated naïve B cells *in vitro* with recombinant BLC (Fig. 17). Strikingly, BLC induced a dose sensitive upregulation of LTα1β2 on cultured B cells after six hours of stimulation (Fig. 17 a). The induction was inhibited by preincubation of the cells with PTX (Fig. 17 b). To examine the specificity of this effect, three other chemokines with chemotactic activity on naïve B cells were tested: the broadly expressed CXCR4 ligand, SDF1; and SLC and ELC, two related CC chemokines made in the T cell area that are

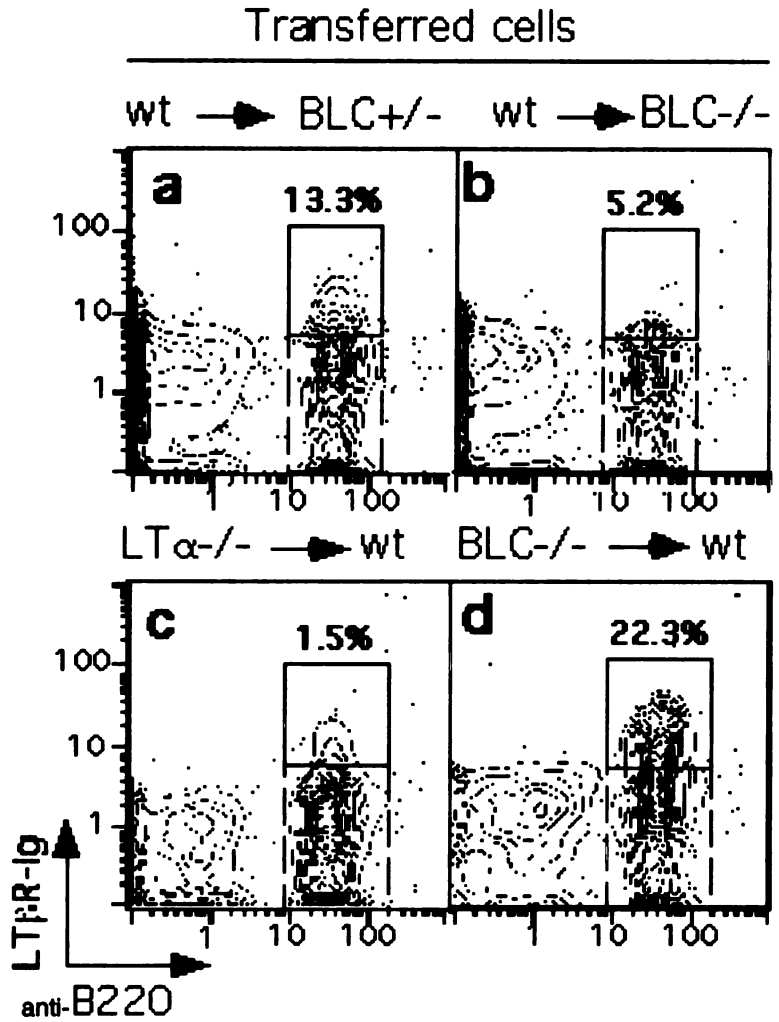

Figure 16. LTα1β2 upregulation on transferred B cells is BLC dependent. Each plot shows flow cytometric analysis of transferred cells in recipient spleen at 6 hrs after transfer. **a,** Transfer of Ly5.1$^+$ wildtype spleen cells into BLC$^{+/-}$ recipients. **b,** Transfer of Ly5.1$^+$ wildtype spleen cells into BLC$^{-/-}$ recipients. **c,** Transfer of Ly9.1$^+$ LTα$^{-/-}$ spleen cells into Ly9.2$^+$ wildtype recipients. **d,** Transfer of Ly9.1$^+$BLC$^{-/-}$ spleen cells into Ly9.2$^+$ wildtype recipients. Data shown are LTβR-Ig and B220 staining of transferred Ly5.1$^+$ (**a, b**) or Ly9.1$^+$ (**c, d**) cells in recipient spleens and are representative of three transfers of each type. Percentages of cells that are LTβR-Ig^{hi} are shown above the windows.

ligands for CCR7 [5]. SDF1 caused an increase in LTα1β2 expression, though the maximal induction was always lower than the maximal induction by BLC (Fig. 17 c and data not shown). By contrast, ELC and SLC caused minimal LTα1β2 upregulation (Fig. 17 c). BLC mediated induction of LTα1β2 was sensitive to actinomycin D, cycloheximide and wortmannin (Fig. 17 d) providing evidence that BLC may signal via a G-protein coupled PI3-kinase to induce lymphotoxin transcription.

DISCUSSION

These studies provide new insight into the mechanism by which TNF and LTα/β promote normal compartmentalization of lymphocytes in the white pulp cords of the spleen. The findings extend the previously defined requirement for TNF and LTα/β in the development and function of follicular stromal cells to also include stromal cells in the splenic T zone. Here we demonstrate that a key function of the LTα/β and TNF-dependent stromal cells is constitutive production of chemokines that strongly attract resting lymphocytes, and they suggest that these chemokines function with other properties of the stroma to compartmentalize cells into follicles and T zones. In addition, our findings indicate that the chemokine BLC can induce increased LTα1β2 expression on the recruited B cells, thus establishing a positive feedback loop between BLC and LTα1β2 that is likely to be critical in follicle development.

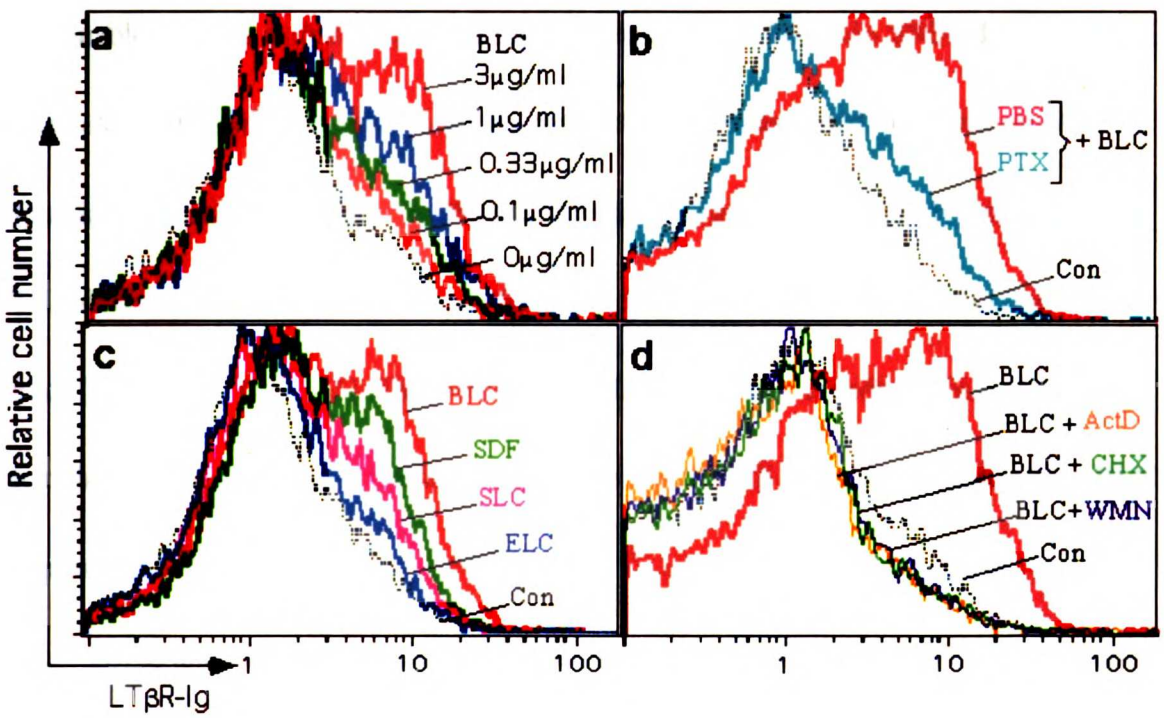

Figure 17. BLC induces LTα1β2 up-regulation on B cells *in vitro*. **a-d,** Histograms show LTβR-Ig staining of B220-gated B cells from *in vitro* cultures in the absence (Con) or presence of chemokines at the indicated amounts. In **b**, cells were pre-incubated with 200 ng/ml PTX or an equal volume of buffer for 2 hours and then incubated with BLC (3 μg/ml) for a further 6 hours. In **c**, cells were incubated in SDF1, SLC or ELC 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 hours.

The chemokine receptor CXCR5 is expressed by all mature B cells and is the only known receptor for BLC, an efficacious attractant of resting B cells [10, 11, 90]. Since loss of CXCR5 is sufficient to disrupt organization of splenic follicles [25], it is reasonable to suggest that the greatly reduced expression of BLC in TNF-, TNFR1-, LTα-, and LTβ-deficient mice directly contributes to the disrupted organization of splenic follicles in these animals. Polarized follicles also fail to form in lymph nodes of TNF-deficient mice and in the nodes that develop under some conditions in LT-deficient mice [38, 42, 43, 105, 115]. The finding that BLC expression is reduced in mesenteric lymph nodes of LTβR-Ig-treated mice indicates that LTα/β plays a role in directing BLC expression in lymph nodes.

SLC and ELC both stimulate cells through CCR7, a receptor expressed by T and B lymphocytes, and these chemokines are the most efficacious attractants of T cells so far described [16, 19, 123] (also see Chapter Two). We propose that the severe reduction in T zone SLC expression in LTα/β-deficient mice directly contributes to the loss of normal T cell compartmentalization in these animals. Maturing DCs upregulate CCR7 and have been suggested to migrate to lymphoid tissues in response to CCR7 ligands [124], making it possible that the decrease in SLC also leads to reduced accumulation of mature DCs in the T zone. Consistent with this possibility, lymph nodes developing in mice with reduced LTα levels have threefold fewer DCs than controls [125], and we observed a

similar decrease in DC frequency in LTα-deficient mouse spleens (our unpublished observations). Fu and coworkers have also recently observed a decrease in DC frequency in $LT\alpha^{-/-}$ mouse spleens [126]. The lowered ELC levels are likely to exacerbate the effect of SLC deficiency and contribute to the loss of T zone organization. TNF and TNFR1 are also required for maximal SLC and ELC expression. However, mice deficient in TNF or TNFR1 do continue to express significant amounts of SLC and ELC, and this is consistent with the relatively unaffected T zone organization in these mutant animals [36-38, 127]. The generally greater reduction of chemokine expression in TNF-deficient compared with TNFR1-deficient mice should not be due to background gene effects, since all the animals were generated on the C57BL/6 background; a more likely possibility is that TNFR2 transmits some of the TNF signals necessary for chemokine expression. Requirements for development of T zone stromal cells have been less well characterized than for FDCs, but our results indicate they are similar in being TNF and LTα/β dependent. Chapter Four will describe in detail further findings regarding the cellular and molecular regulation required for the development of the splenic T zone.

At this stage, it has not been possible to determine whether LT and TNF work directly to induce chemokine expression or whether they function further upstream, inducing and maintaining the development and viability of chemokine-expressing stromal cells. Although treatment of adult mice with soluble LTβR-Ig leads within 1 wk to

decreased expression of BLC and SLC, the treatment also leads to rapid disruption of stromal cells as defined by a variety of markers [104] (also see Fig. 11). Future studies must define in more detail the subpopulations of LT- and TNF-dependent stromal cells that express BLC and SLC and characterize the signaling pathways that control chemokine expression.

The deficiency of FDCs in LT- and TNF-deficient mice has been well characterized [94]. Ultrastructural studies have demonstrated that FDCs are part of a broader network of follicular stromal cells [128], and using the molecule BP-3 as a marker it has been possible to show that this more extensive stromal cell network is also LT and TNF dependent (see Fig. 11). Elegant bone marrow chimera and adoptive transfer experiments have established that FDC development requires TNFR1 and LTβR expression by the follicular stroma and cytokine (LT and TNF) expression by hematopoietic cells, in particular B cells [44, 45, 108, 115]. The necessity for B cells in the maximal expression of BLC (see Fig. 13) is consistent with these results and suggests that a feedback loop exists which helps to keep the number of BLC producing follicular stromal cells in proportion to the number of B cells. In support of this possibility, our data have indicated that the chemokine BLC can induce B cells to upregulate LTα1β2 expression. The positive feedback loop between BLC and LTα1β2 thus causes the low amounts of BLC that are expressed independently of LTα1β2 (see Fig. 9) 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 [129]. A very early step in development of Peyer's patches (PPs) and LNs, is local accumulation of $CD3^-IL7R^+$ cells that express LTα1β2 [130]. As some of these cells express CXCR5 [130] and both CXCR5 and BLC are required for development of PPs and many LNs [25, 100], we propose that BLC helps in recruiting $CXCR5^+CD3^-IL7R^+$ cells and inducing them to upregulate LTα1β2. Consistent with this, it has recently been reported that BLC is highly expressed in the Peyer's patch anlagen [131].

Our studies have established a major role for LTα/β, and a lesser but significant role for TNF, in promoting the function of chemokine-expressing stromal cells in lymphoid areas of the spleen. Analysis of lymph nodes from LTβR-Ig-treated mice has provided initial evidence that LTα/β is also required for normal chemokine expression in lymph nodes. Given the requirement for LTα/β and TNF in normal organization of all peripheral lymphoid tissues, as well as the ability of ectopically expressed LTα to

promote accumulation of B and T lymphocytes in lymphoid aggregates [132], it appears likely that LTα/β and TNF function broadly in regulating lymphoid tissue chemokine expression. Importantly, our proposed novel mechanism of a chemokine-driven positive feedback loop that organizes lymphoid follicles is likely to be critical in lymphoid tissue development. In several human diseases, including rheumatoid arthritis and type I diabetes, ectopic development of follicle- and T zone-like structures is likely caused by locally produced LT and TNF that induce the development of BLC- and SLC-expressing stromal cells. This accumulation of lymphocytes at non-lymphoid sites is reminiscent to that of normal lymphoid tissue development and deserves further investigation in light of our findings.

ACKNOWLEDGEMENTS

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CHAPTER FOUR

SPLENIC T ZONE DEVELOPMENT IS B CELL DEPENDENT

ABSTRACT

The factors regulating growth and patterning of the spleen are poorly defined. We demonstrate here that spleens from B-cell deficient mice have seven- to ten-fold reduced expression of the T zone chemokine, SLC. Expression of the T zone stromal marker, gp38, is also reduced and T cell and dendritic cell numbers are two- to three-fold lower in B cell deficient than in wild-type spleens. B cell deficiency did not affect these parameters in lymph nodes. Using bone marrow chimera approaches, we provide evidence that B cells play an early postnatal role in promoting spleen T zone development. LTα1β2, a cytokine required for SLC expression, is also needed for gp38 expression in the spleen although it is not required for accumulation of normal numbers of T cells. Introduction of a B cell-specific LTα transgene onto the LTα-deficient background restored SLC and gp38 expression, establishing that B cells promote development of T zones, as well as follicles, at least in part by acting as a source of LTα1β2. We suggest that defective spleen development may contribute to the immune defects associated with B cell deficiency in mice and humans.

INTRODUCTION

The spleen participates in immune responses against many types of pathogens [75, 133, 134] and it is involved in multiple autoimmune diseases [93]. Within the spleen, lymphocytes are organized as sheathes around arterioles, with the T zone located centrally (also called the periarteriolar lymphoid sheath or PALS) and the B cells distributed around the T zone in tightly packed follicles. The spleen contains an additional population of B cells in a compartment that surrounds the follicles, known as the marginal zone [75]. The T zones and follicles of the spleen are commonly referred to as the white pulp cords. The remainder of the spleen, termed red pulp, contains large numbers of macrophages, vascular cells and transiting blood cells. This compartment functions in red cell and immune complex clearance.

Current understanding of the factors that pattern lymphoid organs is limited. Within the B and T cell areas there are specialized chemokine-producing stromal cells, believed to be of mesenchymal origin, that help organize the tissue [10, 16, 17, 21, 78]. Follicular stromal cells produce BLC, a ligand for CXCR5, and both CXCR5 and BLC are necessary for formation of B cell follicles [25, 100]. A subset of follicular stromal cells, known as follicular dendritic cells (FDCs), express complement and Fc receptors and are able to trap immune complexes for presentation to B cells. Normal expression of BLC and development of FDCs is dependent on signals provided by B cells [44, 45]. As described in Chapter Three, maturation of follicular stromal cells depends on a positive

feedback loop between BLC and the cytokine LTα1β2, where BLC mediates recruitment of CXCR5-expressing B cells and induces them to express LTα1β2. LTα1β2 then causes growth and maturation of the stromal cells, leading to increased expression of BLC and upregulation of FDC markers (Chapter Three and [44, 45]). Stromal cells within T cell areas produce the CC chemokines, SLC and ELC [10, 16, 17, 21, 78]. In mice lacking lymphoid SLC and ELC [26], or in animals deficient in the common SLC and ELC receptor, CCR7 [27], there is a severe paucity of T cells and DCs within T zones, while follicles continue to develop [26, 27]. Despite their importance as T zone organizers, little is known about the cellular interactions required for development of T zone stromal cells or for expression of T zone chemokines.

We demonstrate here that splenic expression of the T zone chemokine, SLC, is dependent on the presence of B cells and does not depend on T cells. By contrast, SLC expression in lymph nodes does not require B cells. Gp38-staining revealed a greater than three-fold reduction in the amount of splenic T zone stroma in B cell-deficient mice and flow cytometric analysis showed a two- to three-fold reduction in the number of T cells and DCs. Previous studies established that T zone chemokine expression is strongly dependent on LTα1β2 (see Chapter Three) and we find that appearance of Gp38-expressing cells is also LTα1β2 dependent. Transgenic restoration of LTα1β2 selectively on B cells was sufficient to restore T zone stromal networks and SLC expression in

LTα1β2-deficient mice. Our studies suggest that during spleen development, when B cells are occupying the area destined to become the T zone, they provide LTα1β2 and other signals that are critical for induction of SLC and for stromal cell maturation.

MATERIALS AND METHODS

Animals

LTα$^{-/-}$ mice were generated using C57BL/6 embryonic stem (ES) cells and maintained on a pure C57BL/6 background as described [38, 97]. C57BL/6 μMT, C57BL/6 TCRβ$^{-/-}$δ$^{-/-}$, and C57BL/6 recombination activating gene (RAG)-1$^{-/-}$ mice were obtained from The Jackson Labs. Generation of LTα transgenic mice is described in Chapter Six.

Northern Blot Analysis

10-15 μg of total RNA from mouse spleens was subjected to gel electrophoresis, transferred to Hybond N+ membranes (Amersham Pharmacia Biotech), and probed using randomly primed ^{32}P-labeled mouse cDNA probes of the following types: SLC, bases 1-848 [16] and ELC, bases 1-755 (see Chapter Two). To control for RNA loading, a mouse elongation factor 1α (EF-1α) probe was used. For quantitation, Northern blots were exposed to a PhosphorImager screen for 6 h to 3 d and images were developed using a Storm860 PhosphorImager (Molecular Dynamics). Data were analyzed using

ImageQuant® software (Molecular Dynamics), and chemokine mRNA levels were corrected for RNA loaded by dividing the chemokine hybridization signal by the EF-1α signal for the same sample. Relative expression levels were calculated by dividing the corrected signal for each mutant or treated sample with the mean corrected signal for the wild-type or control treated samples, as appropriate, that were included on each of the Northern blots.

Immunohistochemistry

Cryostat sections (6-7 μm) were fixed and stained as described previously [91] using the following mAbs: rat anti-B220 (CalTag); rat anti-MadCAM-1 (PharMingen); goat anti-mouse 6Ckine (SLC) (R&D); sheep anti-IgD (Pharmingen); and serian hamster anti-gp38 (provided by Andrew Farr, University of Washington, Seattle, WA). Rat IgG antibodies were detected with goat anti-rat-conjugated alkaline phosphatase (AP) (Southern Biotechnology Associates) or donkey anti-rat-conjugated horseradish peroxidase (HRP). Goat IgG antibodies were detected with biotinylated donkey anti-goat IgG (Southern Biotechnology Associates), followed by Vectastain ABC-AP (Vector). Sheep IgG antibodies were detected with donkey anti-sheep IgG-HRP (Jackson ImmunoResearch). Serian hamster IgG antibodies were detected with anti-serian hamster IgG-AP (Jackson ImmunoResearch). Enzyme reactions were developed with conventional substrates for peroxidases (diaminobenzidine/H_2O_2 [Sigma]) and alkaline phosphatase (FAST BLUE/Naphthol AS-MX [Sigma]). Sections were mounted in

crystal mount (Biomeda Corp.) and viewed with a Leica DMRL microscope. Images were acquired on an Optronics MDEI850 cooled CCD video camera (Optronics Engineering) and were processed with Photoshop software (Adobe Systems, Inc.).

Cell transfer

Transfer of bone marrow (BM) cells or splenocytes was performed as previously described [135]. In brief, to obtain BM cells, both femurs from adult mice (8 to 10 weeks old) were removed and cut at both ends. The remaining central portion of the bone was placed in RPMI containing 10% fetal bovine serum, 10 mM Hepes, and antibiotics. BM cells from the femur were flushed out with medium through a 28-gauge needle. The cell suspension was centrifuged at 300g for 6 min and the cell pellet was resuspended in fresh medium. 1-5 $\times$ 10^6 BM cells were intravenously injected into lethally irradiated recipient mice (1000 rads). Recipient mice were maintained with antibiotic water for six weeks before being sacrificed for analysis. Splenocyte suspensions were also prepared in RPMI medium as described by gently pressing the spleen through a 70-μm mesh cell strainer (Fisher Scientific Co.). Red blood cells was lysed using Tris-ammonium chloride. Splenocyte suspensions were then resuspended in serum-free DMEM medium containing antibiotics. 2 $\times$ 10^7 splenocytes in a volume of 0.05 ml were injected into the peritoneal cavity of newborn mice with a 30-gauge needle as previously described [135].

RESULTS

B cells regulate splenic T zone chemokine expression

To examine the cellular requirements for expression of the T zone chemokines, SLC and ELC, we performed Northern blot analysis of spleen and lymph node tissue from B- and T-cell deficient mice. Surprisingly, expression of SLC in spleen was strongly dependent on B cells but not on T cells (Fig. 18 A, B). Splenic ELC expression was only weakly affected by lymphocyte deficiency and showed a slightly greater dependence on B cells than T cells (Fig. 18 A, C). Greatly reduced SLC expression was also evident by immunohistochemical analysis of B cell deficient spleen (not shown). In contrast with spleen, SLC expression in LNs was not affected by B cell deficiency (Fig. 18 E). T cells were also not required for SLC expression in lymph nodes (Fig. 18 E). Similarly, lymph node ELC expression was not dependent on B cells or T cells, although in lymphocyte deficient RAG-1$^{-/-}$ mice, ELC levels were exaggerated, suggesting that ELC mRNA made by non-lymphocytes may be enriched in the RNA pool (Fig. 18 F). Previous studies established that splenic expression of the B zone chemokine, BLC, is dependent on B cells but not on T cells (see Chapter Three). In contrast with SLC and ELC, we found that there is also a requirement for B cells for promoting BLC expression in LNs (Fig. 18 D).

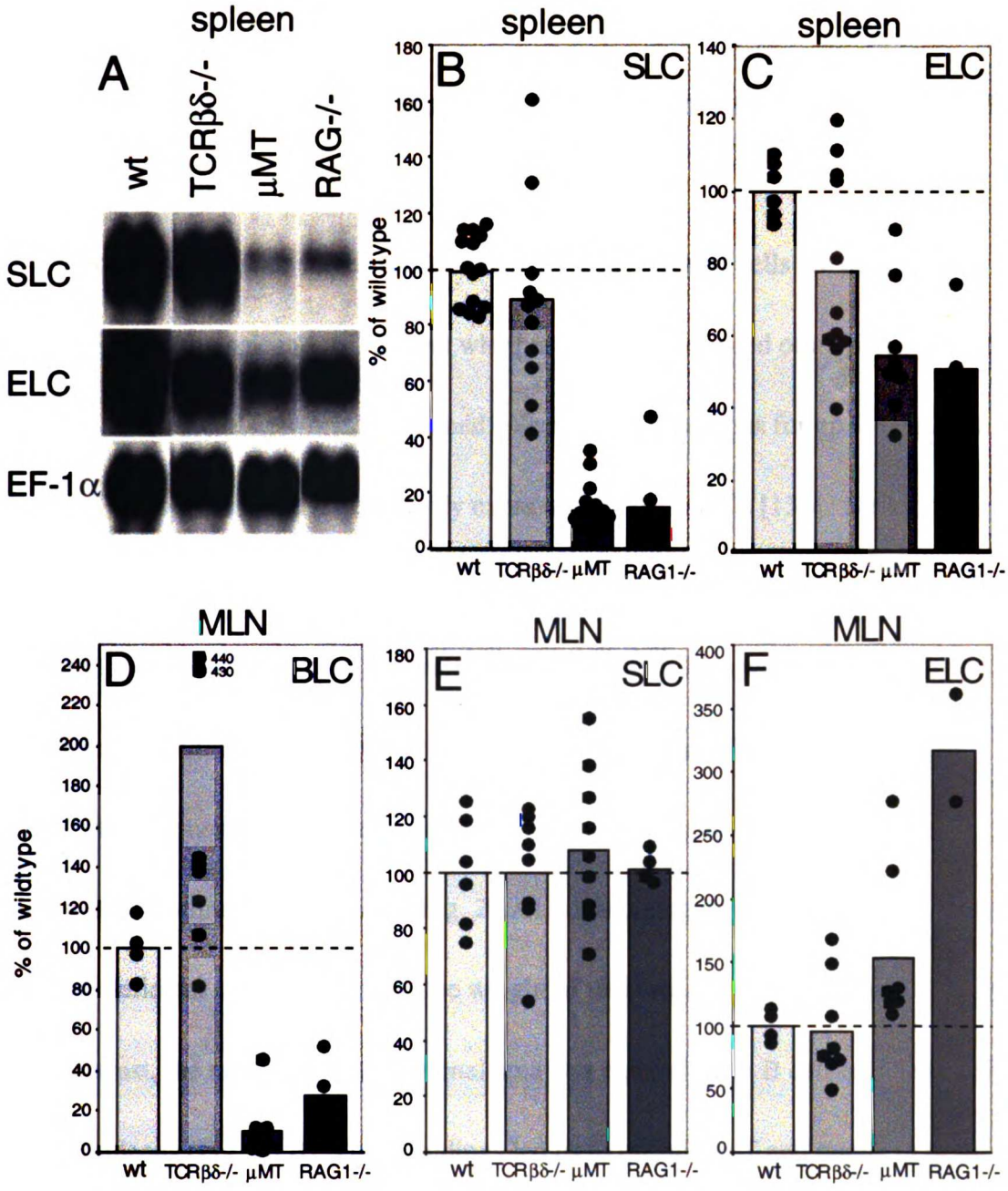

Figure 18. Reduced expression of splenic T zone chemokines in B cell-deficient mice. (A) Northern blot analysis of total RNA isolated from spleen tissue of the indicated mice and probed to detect expression of SLC and ELC. Hybridization to EF-1α was used to control for RNA loaded. WT, wildtype. (B-F) Relative chemokine mRNA levels as determined by PhosphorImager analysis of the Northern blot shown in (A) and additional blots, after correcting for differences in RNA loading from the corresponding EF-1α value. (D-F) Data from mesenteric lymph nodes for BLC (D), SLC (E), and ELC (F). Data from individual mice are shown as filled circles and means as shaded bars.

Requirement of B cells for splenic stromal cell maturation and for normal T cell and DC accumulation in spleen

Recent experiments have established that T zone stromal cells are the major source of SLC in the spleen [16]. To test whether B cells influenced other properties of the T zone stromal cell network, we stained spleen and LN sections for gp38 (Fig. 19), a membrane glycoprotein that is selectively expressed by these cells [136]. Strikingly, there was a three-four fold decrease in the area of gp38 staining in B cell deficient spleen compared to wildtype (Fig. 20 A), whereas gp38 staining in LNs was unaffected by B cell deficiency (Fig. 19). Concordant with the decreased area of T zone stromal cell staining, measurements of T zone cross-sectional area using anti-CD3 revealed an approximate two-three fold decrease (Fig. 20 A). Measurements of spleen weight showed that B-cell deficient spleens were half the weight of their wild-type counterparts (Fig. 20 E). By contrast, the red pulp cross-sectional area was equivalent in B cell-deficient and wild-type spleens (Fig. 20 B). These findings led us to quantitate T cell numbers in the spleen (Fig. 20 C). Strikingly, CD4 and CD8 T cells were reduced by two- to three-fold in B cell-deficient spleens (Fig. 20 C). The number of $CD11c^+$ dendritic cells (DCs) was also diminished (Fig. 20 D). These decreases in T cell numbers were selective to the spleen as B cell-deficient mice had normal numbers of T cells in lymph nodes (Fig. 20 F).

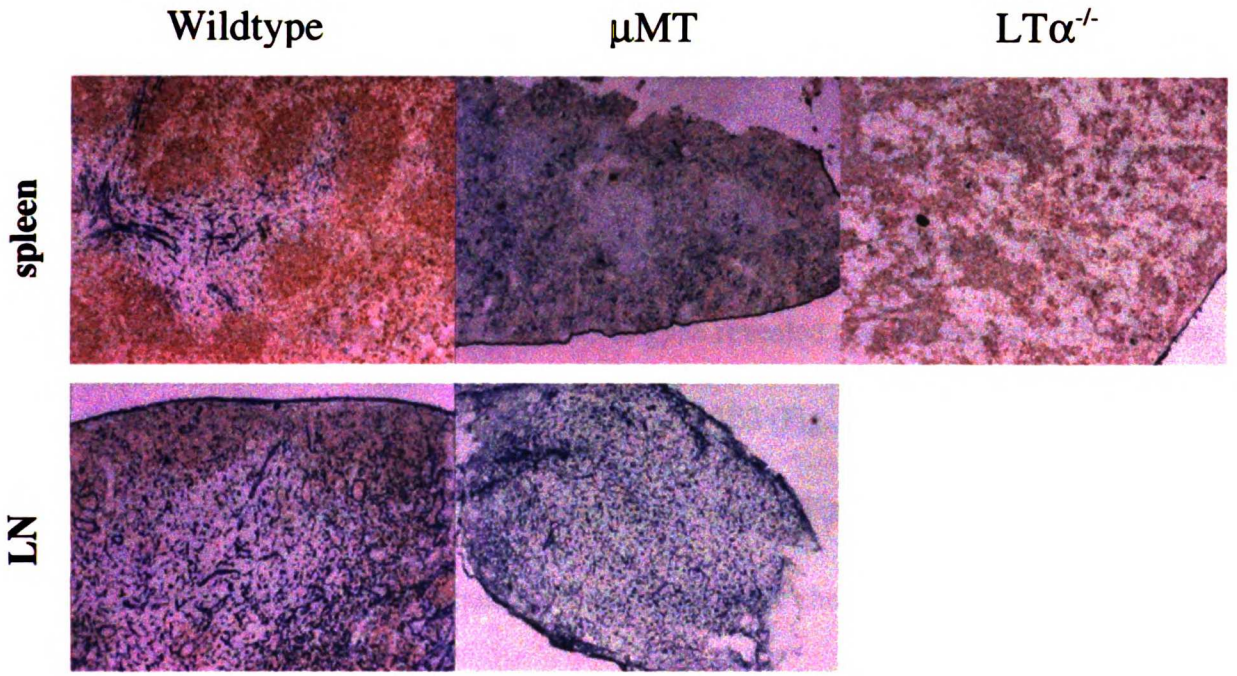

Figure 19. Splenic gp38-staining stromal networks are B cell- and LTα-dependent. Spleen and lymph node (LN) tissue from wildtype, B cell-deficient (μMT) and $LT\alpha^{-/-}$ mice as indicated was sectioned and stained to detect gp38 (blue) and IgD (brown). Original magnification: X5.

Interestingly, T cell numbers in blood were elevated in B cell-deficient mice (Fig. 20 G). In summary, B cells have strong effects on T zone stromal cell development and T cell and DC accumulation in the spleen.

Lymphotoxin is required for splenic T zone stromal cell development

Mice lacking the genes for lymphotoxin (LT) α or β, that are unable to express LTα1β2, have poorly organized T cell areas and are deficient in SLC and ELC expression (see Chapter Three). Analysis of gp38 revealed that expression of this T zone stromal cell marker is strongly dependent on LTα1β2 (Fig. 19). Collectively, these findings established that LTα1β2 is required for splenic T zone stromal cell development. To test whether LTα1β2 is required for maintaining the chemokine producing function of splenic T zone stromal cells, we reconstituted lethally irradiated mice with $LT\alpha^{-/-}$ bone marrow (BM). Consistent with our previous studies, BLC expression was significantly reduced in animals reconstituted with LTα-deficient BM (Fig. 21 A). However, SLC expression was little affected in animals reconstituted with LTα-deficient BM (Fig. 21 B). The failure to observe an effect on SLC expression in the chimeras contrasted with the marked effect of congenic LTα1β2 deficiency and suggested that LTα1β2 plays an important role inducing SLC during spleen development and has a much lesser role in maintaining SLC expression in the adult. In agreement with this, when adult wild-type

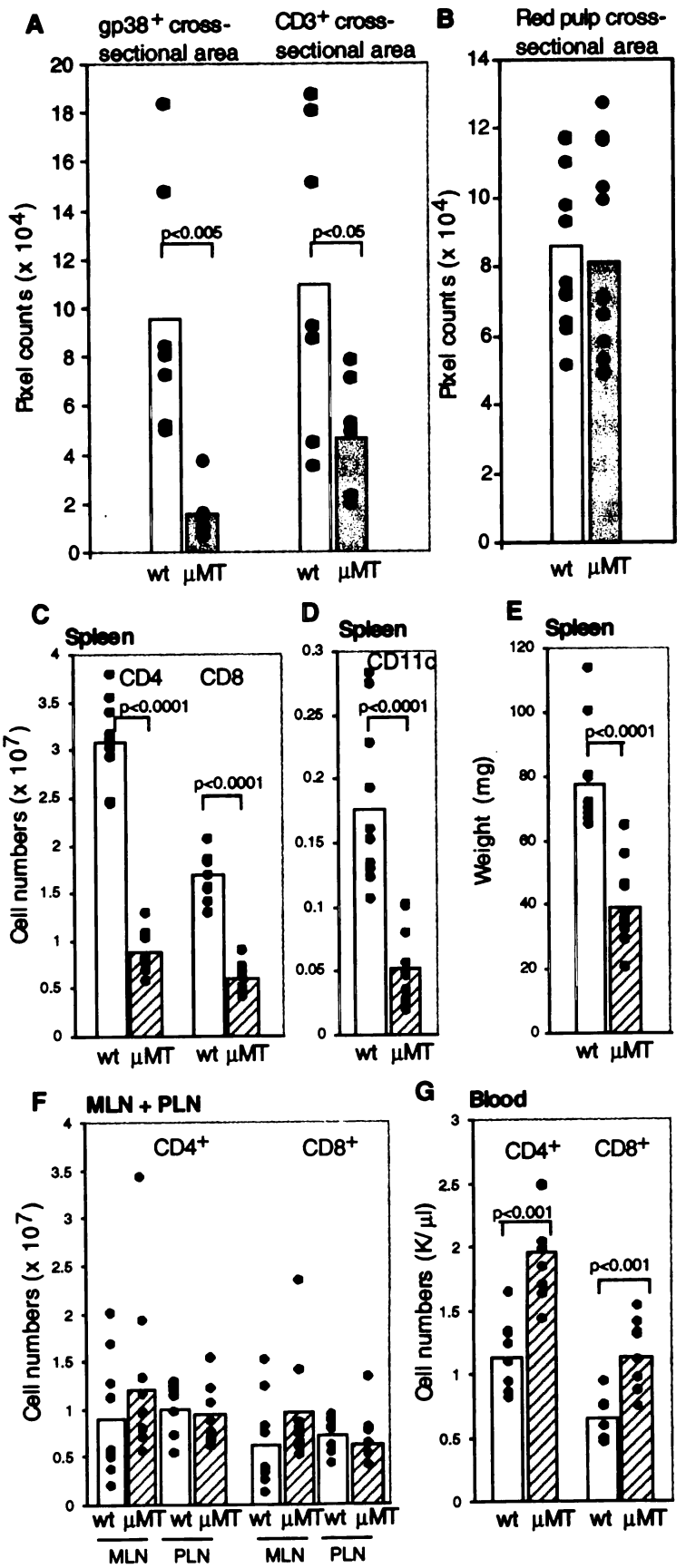
A
gp38+ cross-sectional area
CD3+ cross-sectional area
Pixel counts (x 10^4)
p<0.005
p<0.05
wt µMT wt µMT
B
Red pulp cross-sectional area
Pixel counts (x 10^4)
wt µMT
C
Spleen
CD4 CD8
p<0.0001
p<0.0001
Cell numbers (x 10^7)
wt µMT wt µMT
D
Spleen
CD11c
p<0.0001
wt µMT
E
Spleen
p<0.0001
Weight (mg)
wt µMT
F
MLN + PLN
CD4+ CD8+
Cell numbers (x 10^7)
wt µMT wt µMT wt µMT wt µMT
MLN PLN MLN PLN
G
Blood
CD4+ CD8+
p<0.001
p<0.001
Cell numbers (K/µl)
wt µMT wt µMT

Figure 20. B cells influence splenic T zone stroma and accumulation of splenic T cells and DCs. (A) Splenic T zone gp38-and CD3-staining cross-sectional areas are reduced in μMT spleen. Immunohistochemistry was performed for cross-sections of spleen using anti-gp38 and anti-CD3 antibodies. Staining results were digitally photographed and saved as Photoshop® files. Positive staining areas were selected and the total pixels representing the selected areas are calculated using the Photoshop® software. Each filled circle is a value of total pixels representing total cross-sectional areas that stain positive with the indicated antibodies. Data were collected from three different spleens with more than one cross-section per spleen. (B) Red pulp areas from μMT spleen are similar to that of wild-type spleen. Pixel counts were determined as in (A). The red pulp areas were obtained by subtracting the white pulp areas from the total splenic cross-sections. The white pulp areas of μMT spleen were $CD3^+$ staining areas; and wildtype splenic white pulp areas were combination of $B220^+$ and $CD3^+$ areas. Bar graphs are mean values. (C, D) Reduced T cell and $CD11c^+$ cell counts in μMT mice. Total splenocyte numbers were obtained by counting nucleated cells using a Coulter® counter. Proportions of each splenocyte population were obtained by FACS® analysis using anti-CD4, -CD8, and -CD11c antibodies. Cell counts for each cell population were determined by multiplying the total splenocyte number to the proportion of that population in spleen. (E) Reduced spleen weights in μMT mice. (F) T cell counts are normal in μMT lymph nodes. Data are combined values from mesenteric (MLN) and peripheral (PLN) lymph nodes. (G) Elevated T cell counts in μMT blood. Cell counts were determined as in (C, D) and presented as cell numbers per unit blood volume (x1000/μl or K/μl). Data from individual mice are shown as filled circles and means as open (wildtype) and hatched (μMT) bars. Data shown are from five to ten mice. Statistical comparison is by Student's *t*-test.

mice were reconstituted with μMT or RAG-$1^{-/-}$ BM, SLC mRNA levels remained similar to wild-type (Fig. 21 B, and not shown). Reciprocally, when lymphocyte deficient mice (RAG-$1^{-/-}$) were reconstituted with wild-type BM, they failed to upregulate SLC to wild-type levels (Fig. 21 D). This contrasted with findings for BLC, where substantial upregulation was observed (Fig. 21 C). B lymphocytes begin seeding the spleen soon

after birth [137], and consistent with the ability of B cells to have early post-natal effects, intraperitoneal transfer of B cells to RAG-1$^{-/-}$ mice shortly after birth was sufficient to promote an increase in splenic SLC expression (Fig. 21 E). Importantly, if the transferred lymphocytes lacked LTα, they failed to upregulate SLC (Fig. 21 E). Also consistent with B cells having a direct effect on T zone development in the early post-natal period, B cells were found to be localized in nascent T cell areas during the first few days of life (not shown), a finding in agreement with previous studies [137]. Although the B cells present at this stage of development have an IgD^{lo}CD21^{lo} immature phenotype (not shown), they expressed detectable surface LTα1β2 as assessed by staining cells with LTβR-Ig (not shown). These findings suggested that spatial distribution of B cells during this period and their surface LTα1β2 signals together may be necessary for T zone stromal cell differentiation. Our current studies are investigating whether gp38 and SLC are also expressed during this early post-natal period. In summary, these findings suggested that B cells promote splenic T zone development by acting as a source of LTα. However, the inability in these transfer approaches to fully restore SLC expression left open the possibility that other cell types were important and that B cells were acting by other mechanisms. We therefore used a transgenic approach to test the sufficiency of B cells as a source of LTα1β2 for promoting splenic T zone development.

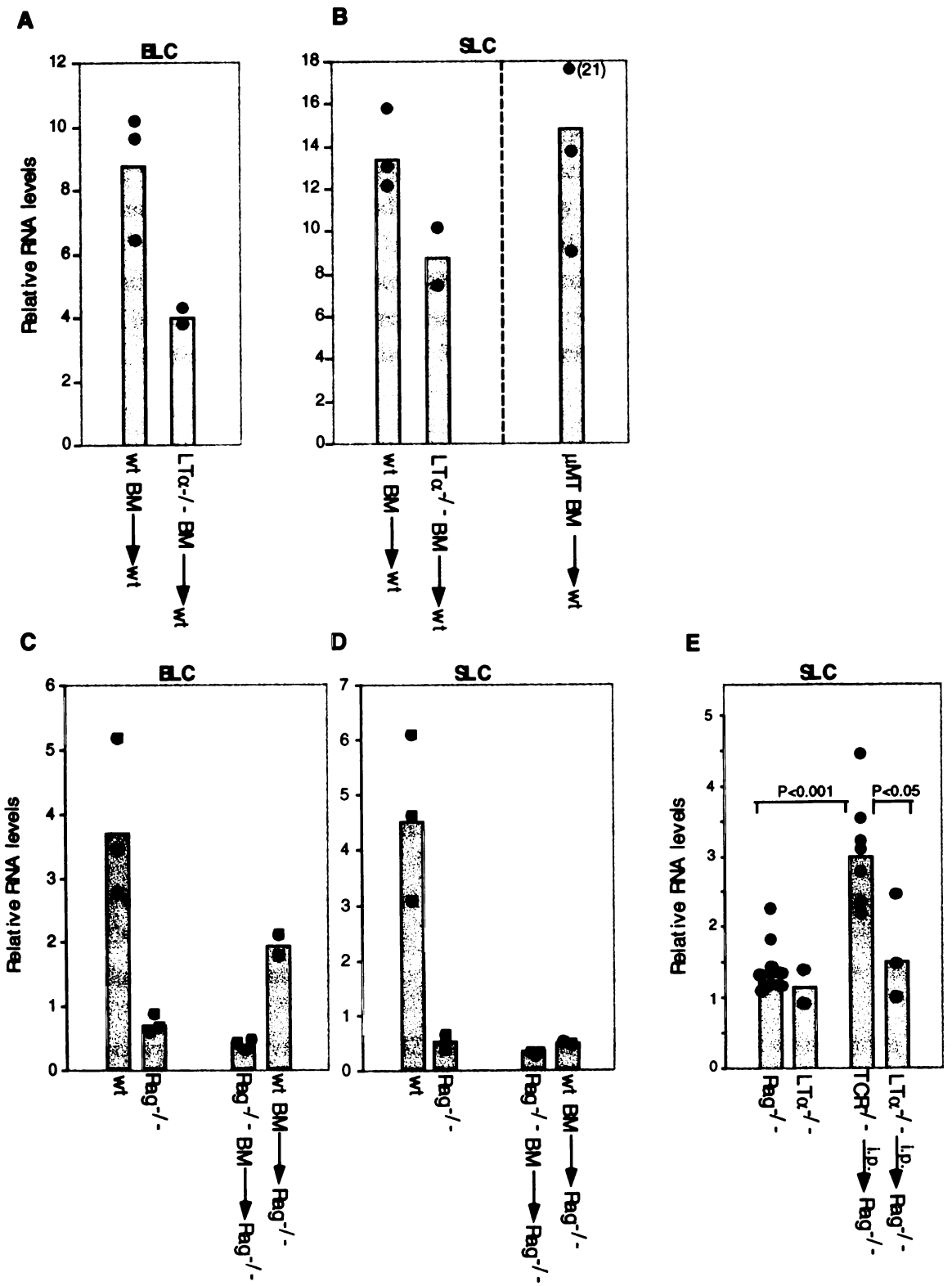
A
BLC
Relative RNA levels
wt BM → wt
LTα-/- BM → wt
B
SLC
(21)
wt BM → wt
LTα-/- BM → wt
μMT BM → wt
C
BLC
Relative RNA levels
wt
Rag-/-
Rag-/- BM → Rag-/-
wt BM → Rag-/-
D
SLC
wt
Rag-/-
Rag-/- BM → Rag-/-
wt BM → Rag-/-
E
SLC
Relative RNA levels
P<0.001
P<0.05
Rag-/-
LTα-/-
TCR-/- i.p. → Rag-/-
LTα-/- i.p. → Rag-/-

Figure 21. B cell and LTα dependence of splenic T zone stroma is developmental. (A-D) Relative chemokine mRNA levels as determined by PhosphorImager analysis of Northern blots. Six weeks after bone marrow (BM) reconstitution to irradiated recipients as indicated, total splenic RNAs were prepared from BM recipients and normal control mice as indicated and were hybridized with radio-labelled BLC (A, C) or SLC (B, D) cDNA. (E) B cells and LTα are required during postnatal development for upregulation of splenic SLC expression. Data shown are relative mRNA expression levels determined as in A-D. 2 x 10^7 Splenocytes from six week-old T cell-deficient mice ($TCR^{-/-}$) and LTα-deficient mice were isolated and intraperitoneally (i.p.) injected into newborn (day 0.5) RAG-$1^{-/-}$ mice. Six weeks after i.p. injection, total splenic RNA was isolated from recipients and control mice as indicated, and hybridized with radio-labelled SLC cDNA by Northern blot. Each filled circle represents an individual recipient and means as shaded bars. Statistical comparison is by Student's *t*-test.

LTα1β2 expression by B cells is sufficient to promote splenic SLC expression and T zone stromal cell development

To generate animals selectively expressing LTα1β2 on B cells, transgenic mice were produced that carry the LTα coding region fused to the LTβ membrane domain, placed under control of the Ig *kappa* enhancer and promoter elements (Fig. 22 A). Previously we have observed that membrane LTα1β2 on B cells is unstable and is rapidly reduced as cells enter circulation (see Chapter Three, Fig. 15 B). Founders were therefore screened for elevated LTα1β2 expression on blood B cells and several were identified that had expression on B cells but not on other cell types. One line was selected that exhibited levels of LTβR-Ig staining on blood B cells that was similar to the level of staining of lymph node B cells in wildtype animals (Fig. 22 B) and this line was

intercrossed with LTα-deficient mice. As expected, the transgenic expression of LTα1β2 on B cells failed to restore lymph node or Peyer's patch development in the LTα-deficient animals (data not shown). However, there was a striking restoration of spleen architecture compared to LTα-deficient animals. T cell areas were present and these contained well developed T zone stromal cell networks (Fig. 22 D). B cell regions were also well developed although they lacked the polarity typical of normal follicles, often appearing as a band of uniform thickness surrounding the T cell area (Fig. 22 D). Marginal zone bridging channels were also poorly developed (Fig. 22 D). In addition, the separation of B and T cell areas was less well demarcated than in wild-type animals. Analysis of chemokine expression revealed strong staining of T cell areas for SLC (Fig. 22 D). By Northern blot, expression of LTα1β2 selectively on B cells restored BLC to wildtype levels and caused SLC to be expressed somewhat above wild-type controls (Fig. 22 C).

DISCUSSION

Twenty years ago it was established that B cells provide signals needed for the development of follicular dendritic cells [44, 138, 139]. In the above studies, we demonstrate that B cells also play a critical role in promoting development of splenic T zone stromal cells. In mice lacking B cells, the T zone stroma fails to develop into its

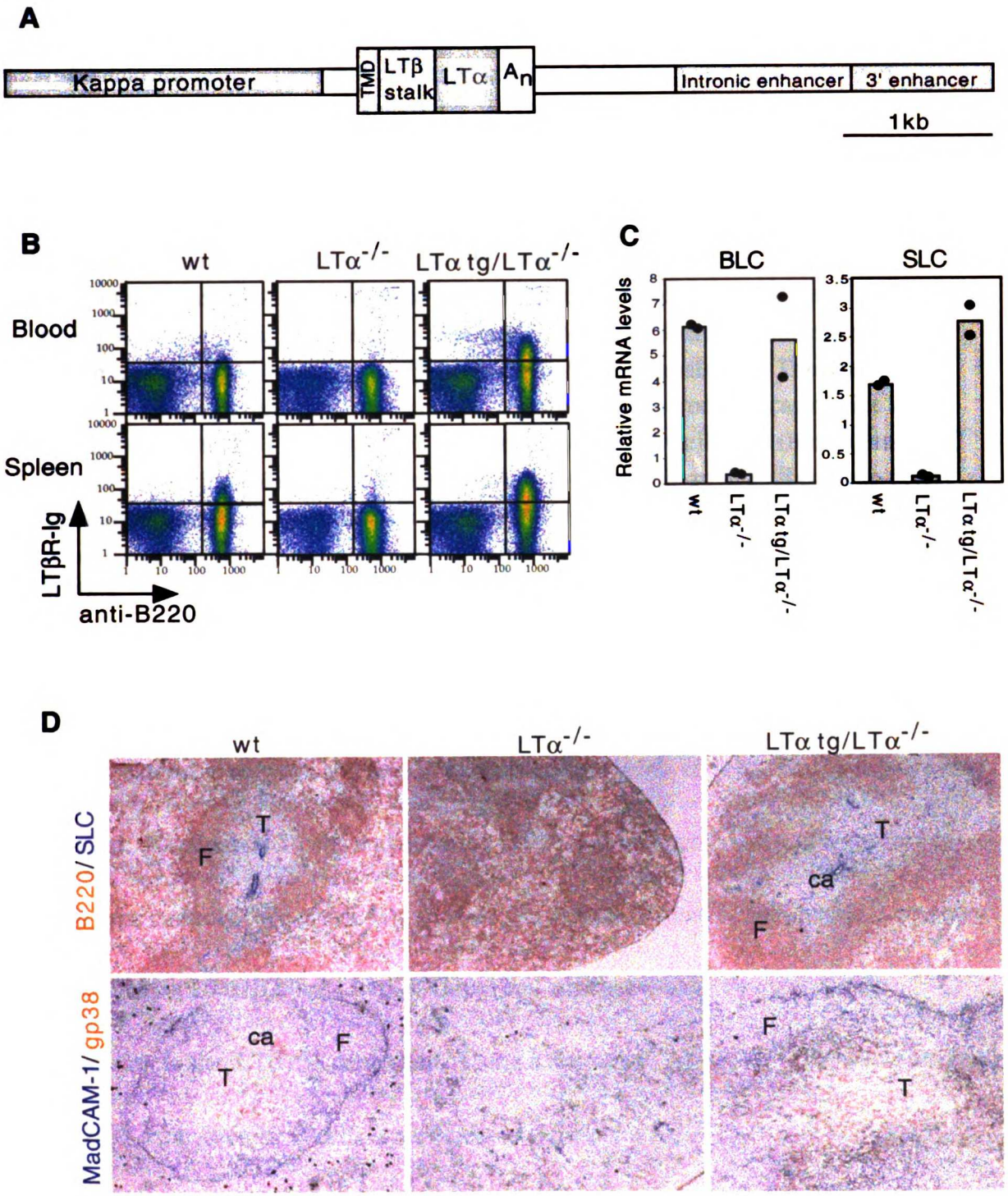
A
Kappa promoter
TMD
LTβ stalk
LTα
An
Intronic enhancer
3' enhancer
1kb
B
wt
LTα-/-
LTα tg/LTα-/-
Blood
Spleen
LTβR-Ig
anti-B220
C
BLC
SLC
Relative mRNA levels
wt
LTα-/-
LTα tg/LTα-/-
D
wt
LTα-/-
LTα tg/LTα-/-
B220/SLC
MadCAM-1/gp38
T
F
ca

Figure 22. Transgenic expression of LTα1β2 by B cells promotes splenic SLC expression and T zone stromal cell development. (A) A schematic diagram of the LTα transgenic construct. The DNA construct contains 2-kb *kappa* promoter element upstream of the chimeric LTβ stalk/LTα cDNA, followed by the late SV40 polyadenylation site and intronic enhancer and 3' enhancer sequences. The transmembrane domain of the LTβ gene is marked as TMD. (B) Flow cytometry profiles of LTβR-Ig binding activitiy of B cells isolated from blood and spleen from one line of transgenic animals (LTαtg/LTα$^{-/-}$) and other control animals as indicated. (C) Restoration of chemokine expression in LTα-deficient mice by expression of the transgene. Data shown are relative splenic mRNA levels from indicated mice as determined by PhosphoImager analysis of Northern blot signals. (D) Transgenic expression restores splenic T zone stroma. Spleen sections from indicated mice were stained with anti-B220 and anti-SLC antibodies to detect B-cell areas (brown) and SLC protein expression (blue) and with anti-MadCAM-1 and anti-gp38 antibodies to detect the marginal zone sinus-lining cells (blue) and T zone stroma (brown), respectively. T, T zone; F, follicle; ca, central arteriole. Original magnification: top row: ×5, bottom row: ×10.

normal extensive network and fails to express normal levels of SLC. ELC levels are also reduced. Numbers of T cells and dendritic cells are significantly reduced compared to wild-type controls. We previously showed that T zone chemokine expression is strongly dependent on LTα1β2 (see Chapter Three) and we demonstrate here that Gp38 expression is also LTα1β2 dependent. Using a transgenic approach, we demonstrate that LTα1β2 expression by B cells is sufficient to restore T zone stromal networks and SLC expression in LTα1β2-deficient mice. Our studies suggest that during the early post-natal period of spleen development, B cells occupy the T-cell area and provide LTα1β2 signals that are critical for induction of SLC and for stromal cell maturation.

The factors regulating lymphoid organ growth are poorly defined. Development of the spleen begins at approximately embryonic day 13 in the mouse and several transcription factors have been identified that play an essential role in early steps of spleen development [140]. B cells begin entering the spleen around birth, when the spleen is at least 10-fold smaller than the adult spleen (unpublished observations) and the first B cell follicles become evident by week 1 of life [137]. Several previous reports have noted that B cell-deficient mice have small spleen size [141] and generally this was considered a reflection of the physical absence of B cells. We have now shown that B cell-deficient animals have a deficiency of splenic T cells, DCs and stromal cells (Fig. 20 A, C, D). An important role for B cells in promoting lymphoid organ growth was recently reported with the finding that Peyer's patch development and growth is severely affected in B cell-deficient mice [129]. The approximately normal spleen size in mice lacking LTα1β2 indicates that the role of B cells in promoting spleen growth is not through expression of LTα1β2 and, consistent with this, transferred LTα-deficient B cells promoted spleen organ growth as efficiently as wild-type B cells (unpublished observations). It is possible that LTα1β2 has a dominant effect on chemokine expression and cell accumulation in the splenic white pulps; whereas, accumulation of cells in the red pulp is independent of LTα1β2. We propose that B cells act as a source of a factor, perhaps an FGF or TGF family member, that promotes growth of splenic mesenchymal

cells. As the populations of stromal cells expand, they may accommodate more recirculating hematopoitetic cells.

B cell-deficient mice have a two-fold decrease in spleen cellularity and mass, whereas SLC expression is decreased at seven-fold. This disproprotional effect of SLC expression suggests that B cells have additional influences on the splenic T zone compared to their effects on other regions of the spleen. By several approaches, we discovered that the role of B cells in promoting SLC expression is developmental, and once mice had developed with a normal spleen, LTα plays only a small role in maintaining SLC. Furthermore, reconstitution of irradiated adult RAG-$1^{-/-}$ or μMT mice with wildtype BM did not upregulate SLC expression, suggesting that T zone stromal cells in these mice are unresponsive to SLC-inducing signals from B cells. This developmental role of B cells in promoting SLC expression and differentiation of splenic T zone stromal cells was supported by observations of B cell distribution around the periarteriolar regions destine to become T cell areas. B cell localization within this nascent T zone during the first few days after birth may therefore allow B cells to interact and provide LTα1β2 signals for maturation of the T zone stroma, including upregulation of SLC expression. In support of the early temporal requirement of LTα1β2 for SLC expression, transfer of wild-type B cells into newborn RAG-$1^{-/-}$ mice was sufficient to upregulate SLC expression. However, transfer of LT$\alpha^{-/-}$ B cells failed to induce a

detectable increase of SLC levels. The sufficiency of B cells as a source of LTα1β2 for promoting splenic T zone development was further established by the demonstration that transgenic expression of LTα1β2 can rectify multiple defects of splenic architecture in LTα-deficient mice, including restoration of T zone stromal networks and SLC and BLC expression.

In contrast to the requirement for B cells in development of spleen and Peyer's patches, development of LNs appears unaffected by B cell deficiency. The explanation for this discrepancy is unknown but it is interesting that spleen and PPs are B cell-rich lymphoid organs whereas LNs are dominated by T cells. However, LN development does not appear strongly dependent on T cells, making it unclear what type of cellular interaction is needed for promoting LN growth.

Our findings in this study strongly indicate important roles for B cells in controlling spleen organ growth and the development of T cell areas. The results of B cell-derived LTα1β2 that is required for splenic SLC expression during early development provides a molecular basis for understanding intercellular communication between cells of the immune system. Our results also have implications for understanding and interpreting findings from numerous studies that have used B cell-deficient mice as a model system to study T cell immune responses.

ACKNOWLEDGEMENTS

We thank Richard Cornall (Oxford University, Headington, UK) for generously providing the Ig kappa promoter/enhancer pBXD1 construct, Andrea Schmidt for excellent help in microinjection, and Andrew Farr (University of Washington, Seattle, WA) for the gp38 antibody.

CHAPTER FIVE

CONCLUSIONS

These studies have examined the molecular mechanisms underlying two important aspects of secondary lymphoid tissue function: coordinated movements of lymphocytes into appropriate compartments during homeostasis and immune response and cellular interactions between lymphocytes and stromal cells that are essential for lymphoid tissue compartmentalization. Three main results have been described in this dissertation. First, murine ELC, which is expressed by cells in the T zone, is a strong chemoattractant for naïve T cells, maturing DCs and antigen-binding B cells. This chemokine is likely to play an important role in T cell and DC homing into the T zone and repositioning of antigen-activated B cells into the outer T zone. Second, the B-cell homing chemokine, BLC, is shown to function not only as a chemoattractant, but also to induce cells to upregulate LTα1β2, a cytokine required for follicular stromal cell development and BLC expression. The existence of a positive feedback loop between LTα1β2 and BLC helps explain how follicles develop and how homeostasis between B cells and follicular stromal cells is maintained. Finally, B cells, through the LTα1β2 signal and possibly additional signals, regulate the development of splenic T zone stromal cells and their production of chemokines that are necessary for compartmental homing of T cells. Interpretations and implications of these results have been discussed in each

chapter and will be mentioned again here only when relevant. Here, I will discuss the connections of my results with findings from other investigators. Future directions pertaining to each chapter that I would like to see pursued will also be presented.

ELC mediates T cell homing into the T zone and relocation of antigen-binding B cells to the outer T zone

Rare antigen-specific T cells must detect the corresponding antigen presented on DCs so that they can initiate an adaptive immune response [3, 47, 48]. The T zone of secondary lymphoid tissues functions as a meeting place for T cells and DCs [1]. It has been speculated that both T cells and DCs may respond to the same attractive cues in the T zone. Our findings that ELC is expressed by cells in the T zone and strongly attracts naïve T cells have provided a molecular basis for T cell homing into the T zone. More recently it has been shown that ELC and SLC are both expressed by T zone stromal cells [78]. Lower amounts of ELC are made by DCs, especially $CD8^+$ DCs [78]. In the case of DC migration into the T zone, previous studies of Langerhans' cell migration from the skin have indicated that, as they begin to mature, DCs up-regulate CCR7 and become responsive to ELC and SLC [142-144]. Thus, these results agree with the prediction that both T cells and DCs respond to the same chemokines (ELC and SLC) and that this serves as a mechanism to promote their encounter in the T zone. Together, these studies

have suggested that ELC and SLC-producing T zone stromal cells play an important role in bringing T cells and DCs together to initiate adaptive immunity.

In our findings, the weak ELC responsiveness of both immature and mature B cells supports a role for ELC in attracting B cells into the T zone as the first step before they migrate into follicles. These results are in agreement with studies by others in which transferred CCR7$^{-/-}$ B cells pass more rapidly through the splenic T zone into follicles [27]. It is not clear, however, whether responsiveness of resting B cells to T zone chemokines is functionally significant as B-cell follicles form normally in CCR7$^{-/-}$ mice and *plt* mice that lack both lymphoid SLC and ELC expression [26, 27].

Previous studies showed that, upon binding antigens, B cells relocate to the outer T zone where they can interact with antigen-specific T cells. This process of follicular exclusion of antigen-binding B cells is thought to promote encounters with helper T cells during immune responses [53, 67, 145]. Our findings that antigen receptor-activated B cells increase their responsiveness to ELC therefore provide a likely molecular mechanism for this relocation of antigen-engaged B cells to the outer T zone. In agreement with our results, earlier findings by others indicated that B cell activation caused increased expression of CCR7 [64, 66]. Future studies with CCR7$^{-/-}$ B cells should be useful to assess the *in vivo* relevance of ELC function in effecting the migratory behavior of antigen-engaged B cells during an immune response.

SLC, a closely related homologue of ELC, is expressed at highest levels by HEV and at lower levels by stromal cells in the T zone [16]. Interestingly, our results and those from other investigators have shown that ELC and SLC have a strikingly similar chemotaxis profile, since both molecules attract naive T cells more efficiently than memory cells, and attract B cells more weakly than T cells (see Chapter Two). Similar to our finding that mouse ELC is a ligand of CCR7, SLC was found to bind to CCR7 [123]. Activated B cells also show increased responsiveness to SLC (V.N. Ngo; J. G. Cyster, unpublished observations). The striking similarity between ELC and SLC in their chemotactic activities and expression pattern suggests that the two chemokines work together to attract T cells into the T zone and coordinate movements of antigen-engaged B cells during immune responses. Future studies should focus on understanding the factors that regulate ELC/SLC expression and the signals that control ELC/SLC responsiveness of lymphocytes. Such studies are likely to provide new insights into how secondary lymphoid tissues function in immunity.

Lymphoid follicle development is dependent on a chemokine-driven feedback loop

In exploring the mechanism of follicle development in secondary lymphoid tissues, we found two important results. First, BLC expression by follicular stromal cells is strongly dependent on B cell-derived LTα1β2 and to a lesser extent on TNF. Second, in addition to mediating B cell attraction, BLC also induces increased LTα1β2

expression on the recruited cells. Together, these findings establish a positive feedback loop between BLC and LTα1β2 that is likely to be critical in follicle development. This positive feedback loop causes the low amounts of BLC that are expressed independently of LTα1β2 (Fig. 9, Chapter Three) to become upregulated; this may help to expand the follicular compartment to accommodate more recruited B cells. Expansion of the follicular compartment is likely to occur in the development of lymphoid follicles or during an immune response when the number of B cells entering a lymphoid organ increases (Fig. 23).

Our findings have identified a novel, homeostatic function of a chemokine that contrasts with previously known effector functions of chemokines studied in the context of inflammatory responses. The effects of chemokines on physiological changes of leukocytes are mostly mediated by seven-transmembrane receptors coupled to $G_{\alpha i}$ subfamily members of *pertussis* toxin-sensitive GTP-binding proteins. These studies, notably of the IL-8 receptor, have shown that, upon ligand binding, $G_{\alpha i}$ protein-coupled receptors initiate a cascade of signaling events leading to activation of a phosphatidylinositol-specific phospholipase C, phosphotidylinositol-3-OH kinases, small GTPases, protein kinase B and Src-related tyrosine kinases [24, 55, 146]. Phospholipase C enzymatic activity leads to activation of two main downstream events: (i) increased intracellular calcium concentration that, in neutrophils, is essential for granule release and

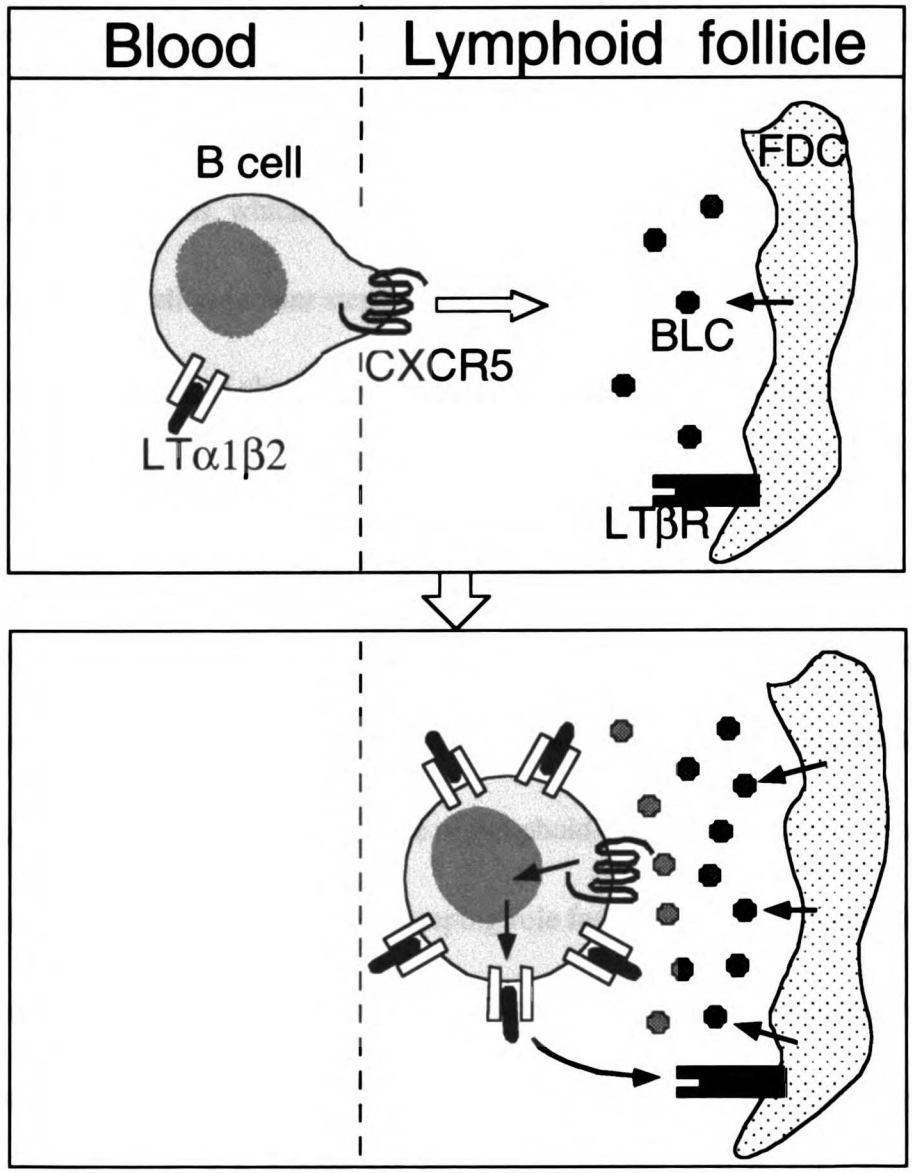

Figure 23. Model of the proposed dual roles of BLC in promoting lymphoid follicle development: chemoattraction of $CXCR5^+$ cells and induction of increased $LT\alpha1\beta2$ expression on the recruited cells. By signaling through LTβRs on follicular stromal cells, a positive feedback loop is established that leads to increased BLC expression and maturation of FDC.

superoxide production and (ii) protein kinase C-mediated effects. Small GTPases induce cellular shape changes through cytoskeletal rearrangements, changes that are necessary for adhesion and chemotaxis [147, 148]. Chemokine binding also induces upregulation and activation of integrins, which function in the adhesion of circulating leukocytes to endothelial cells as well as in later steps during cell migration into the tissue [79]. Other pro-inflammatory effects of chemokines on recruited leukocytes include inducing the release of IL-4, which increases IgE production, and IL-5, which activates eosinophils and basophils [149]. In contrast with these inflammatory functions of chemokines, our results revealed a novel role for BLC. In addition to its chemotactic function typical of a chemokine family member, it can induce naïve B cells to express LTα1β2, a cytokine required for development and maintenance of lymphoid follicle architecture. This finding has therefore established a housekeeping role for BLC in the homeostasis of B-cell compartments that in turn is important for B-cell immune surveillance and antibody production. To further understand this homeostatic role of BLC in the process of follicle formation, future studies are needed to dissect intracellular signaling mechanisms downstream of the BLC receptor, CXCR5, which is responsible for upregulation of LTα1β2 expression. In this context, our initial studies have suggested that the transcriptional regulation of LTα1β2 expression downstream of CXCR5 is at least in part dependent on phosphotidylinositol-3-OH kinases (Fig. 17 d). Studies by other

investigators also began to provide insights into the intracellular signaling pathway involved in the development of lymphoid tissue architecture. Notably, studies of the spontaneous mutant alymphoplasia (*aly*) mice, which carry a point mutation in the gene that encodes nuclear factor *kappa*B–inducing kinase (NIK), have provided evidence that NIK is involved in signaling transduction of seven-transmembrane chemokine receptors expressed by hematopoietic cells [150]. NIK has also been shown to function downstream of LTβR expressed by stromal cells [151]. Animals that lack NF-*kappa*B family members p100/p52 and bcl-3, which are activated by stromal cell-associated TNFR1 and LTβR, also have defects in development of B-cell follicles and BLC expression [152-156]. Further studies are needed to precisely define the cell type(s) expressing LTβR and TNFR1 and to understand how signals from these receptors promote stromal cell development and chemokine productions.

In keeping with the concept that lymphoid tissue chemokines have a homeostatic role, as demonstrated in our studies by the ability of BLC to induce LTα1β2 expression, SDF-1, another constitutively expressed lymphoid tissue chemokine, has been shown to have a homeostatic role in B cell development [12, 62]. SDF-1 not only has a strong chemoattractant activity for B-cell precursors, it also functions as a growth factor for the maturation of these cells in the bone marrow [12, 62]. SDF-1 may also function more broadly in the development of non-lymphoid tissues as SDF-1-deficient mice have a

defect in heart ventricular septum formation [61]. Interestingly, just as we have shown that signaling through CXCR5 induces expression of LTα1β2, other studies have indicated that signaling through CXCR4 can upregulate Fas/CD95 ligand, another TNF family member [157, 158]. These latter findings have provided another interesting analogy that may indicate a similarity between signaling events downstream of CXCR5 and CXCR4, both of which belong to the CXC-type chemokine receptor family and lead to expression of TNF family members.

The concept of positive feedback between molecules expressed by adjacent cells that are in cognate interaction has been frequently encountered and experimentally demonstrated in different developmental contexts. For example, in the development of vertebrate limbs, the outgrowth of limb buds requires the fibroblast growth factor (FGF)-4 signal which is expressed by cells in the apical ectodermal ridge (AER) [159, 160]. This limb bud outgrowth occurs in conjunction with limb patterning that is governed by sonic hedgehog (SHH) signals made in the zone of polarizing activity (ZPA) posterior to the AER [161, 162]. FGF-4 and SHH have been shown to regulate the expression of each other, establishing a positive feedback loop that coordinates growth and patterning in the limb [163]. Our discovery of a chemokine-mediated positive feedback loop has therefore extended this concept to the formation of compartments in lymphoid organs.

As described above, our studies suggest that an initial small amount of BLC expressed by stromal cells attracts early B cells and induces them to produce LTα1β2.

This in turn stimulates stromal cells to differentiate, expand, and express more BLC to accommodate more entering B cells. However, the size of normal lymphoid follicles is always maintained with BLC expression levels proportional to the numbers of B cells. Very little is known about the process that down-regulates this positive feedback loop of BLC and LTα1β2. It is possible that, upon binding ligands, either the receptor CXCR5 or LTβR, or both, are down-regulated by endocytosis. However, in the course of our studies, we discovered that the heterotrimer, LTα1β2, expressed on the surface of B cells is extremely unstable (see Chapter Three). Since the heterotrimer is composed of two subunits of the membrane-bound LTβ that is non-covalently attached to the secreted form of LTα [164], it is possible that the LTα rapidly dissociates, thus giving the complex a short half-life. Alternatively, LTβ may be cleaved from the membrane by a metalloproteinase, analogous to the way TNF is cleaved [165, 166]. Future studies should determine whether the dissociation of the LTα1β2 complex helps to limit the actions of the BLC-LTα1β2 positive feedback loop to sites where BLC is produced.

As briefly discussed in Chapter Three, the role of BLC and LTα1β2 in lymphoid follicle formation described in our studies is also implicated in the process of lymph node organogenesis. Mice deficient in CXCR5 or BLC lack most lymph nodes and have defective Peyer's patch development [25, 100]. In developing lymph nodes and Peyer's patches, several investigators have identified an unusual population of small, bone

marrow-derived $CD3^-IL7R^+$ cells that express $LT\alpha1\beta2$ and CXCR5; they proposed a lymphoid organogenic role for these cells [130, 167]. Recent analysis of mutant mice deficient in a transcriptional inhibitor Id2 or an orphan steroid receptor RORγ showed that the mice lack $CD3^-IL7R^+$ cells. These mice also lack lymph nodes and Peyer's patches, supporting the organogenic role for these cells [168-170]. We propose that BLC functions in recruiting $CXCR5^+CD3^-IL7R^+$ cells and inducing them to upregulate $LT\alpha1\beta2$, which causes a cascade of downstream effects essential for forming and organizing a new tissue. Furthermore, in a transgenic mouse strain that ectopically expresses BLC under the insulin promoter, lymph node-like structures develop in the pancreatic islets in a $LT\alpha1\beta2$-dependent manner [171]. In summary, it appears likely that the positive feedback loop between BLC and $LT\alpha1\beta2$ may function broadly to regulate the development of lymphoid follicles, secondary lymphoid organs, and ectopic lymphoid-like structures in chronic inflammatory conditions, including rheumatoid arthritis and type I diabetes. Future understanding of how this positive feedback mechanism is regulated and whether similar pathways exist for other chemokines and other members of the TNF family, such as TRANCE [172, 173], will provide insights into how lymphoid tissues are formed under normal or disease conditions.

B cells regulate splenic T zone development

The requirement of B cells for the development of follicular dendritic cells has been well established [44, 138, 139]. The results presented in Chapter Four indicate that B cells also play a critical role in promoting development of splenic T zone stromal cells. B cell-derived LTα1β2 has been shown to be required for the development and maintenance of B cell follicles [44, 45]. Similarly, our results also showed that B cells can function as a sufficient source of LTα1β2 to promote T zone stromal development. However, unlike the continuous requirement of B cell-derived LTα1β2 signals for the maintenance of lymphoid follicle structures, B cells appear to influence the splenic T-cell area only during a short post-natal period of spleen development.

The finding that B cells are required for normal splenic expression of SLC and ELC on the T zone stromal cells led us to examine whether accumulation of T cells in the T zone is also defective in the absence of B cells. We found that T cell-staining areas and T cell accumulation in the spleen were significantly reduced in B cell-deficient mice, suggesting that several properties of T cell compartments are B cell-dependent. However, our data showed that SLC expression in the adult spleen is not dependent on the continuing presence of B cells, as wild-type spleen reconstituted with B cell-deficient BM still contains wild-type levels of SLC expression. These findings suggest that in adults, B cells no longer provide the signals necessary for SLC expression by T zone

stromal cells and that the effect seen in B cell-deficient spleen reflects a developmental role of B cells. Previous investigators have shown that in the first few days after birth, splenic B cells occupy the nascent T zone around blood vessels before T cells populate this area [137]. To determine whether the T zone stromal cells in this early stage of development can respond to signals from B cells, we injected B cells into newborn RAG-$1^{-/-}$ mice and found that SLC expression was significantly upregulated. Also in this experiment, in contrast to increased SLC expression in mice that receive wild-type B cells, we did not detect SLC upregulation in mice that received LTα-deficient cells; this indicates that LTα1β2 signals delivered by B cells are necessary for SLC expression during this period. However, the inability in these transfer approaches to fully restore SLC expression leaves open the possibility that other cell types are important and that B cells are acting by other mechanisms. We therefore used a transgenic approach to test the sufficiency of B cells as a source of LTα1β2 for promoting splenic T zone development. As expected, a B cell-specific LTα transgenic line crossed to a LTα-deficient background was able to correct multiple structural defects observed in LTα-deficient spleens, including T zone stromal development and SLC and BLC expression. These findings strongly support the view that LTα1β2 expressed by B cells must be present for the development of T cell areas. To rule out the possibility that overexpression of the

transgene may not reflect physiological levels of LTα1β2, it will be important to repeat these studies in transgenic lines with lower expression of the LTα1β2.

Our results implicate a developmental role for B cells in shaping the splenic T cell area during a brief post-natal period. Since LTα1β2 is continuously required for SLC expression throughout adulthood (see Fig. 10, Chapter Three; Fig. 21B, Chapter Four), we propose the existence of a non-B cell source that continues to provide LTα1β2 and possibly other signals for maintaining splenic SLC expression. The identification of this cell type will be a subject of future studies.

In summary, the studies presented here have provided new insights into the cellular and molecular mechanisms of secondary lymphoid tissue compartmentalization that is essential for immune function. These studies suggest that the T zone chemokine ELC, together with SLC, plays a central role in promoting T cell-DC encounter and B cell relocation during an immune response. These studies have also proposed a mechanism of cellular interactions between B cells and follicular stromal cells. These interactions are driven by a positive feedback regulation between BLC and LTα1β2, which is likely responsible for the formation and for the maintenance of B-cell follicles. B cells and their LTα1β2 signals are also important for the development of T-cell compartments in the spleen. These studies provide a framework for future studies of the

mechanisms that mediate the formation and function of lymphoid tissue compartments. It is hoped that an understanding of the organizing mechanisms of lymphoid tissues will improve our knowledge of immunity in general and of human immunological diseases.

CHAPTER SIX

FREQUENTLY USED MATERIALS AND METHODS

This chapter describes frequently used methods in this dissertation. Although some of these are mentioned in the Materials and methods section of each chapter, more critical observations and important modifications of these methods are further discussed in greater details here.

Isolation of total RNA from lymphoid tissues and cell suspensions

Total RNA from lymphoid tissues and cell suspensions were prepared using the Trizol solution (GibcoBRL) in accordance with the manufacturer's instructions. For isolation of total RNA from tissues, an important modification was made and consistently generates high quality RNA. Briefly, about 20 mg of spleen or lymph node tissues or $5x10^5$-$5x10^6$ cells were completely homogenized with 1ml of Trizol solution in a 2ml-eppendorf tube until no visible chunks of tissue were present. Tissue homogenization was carried out with either a motorized homogenizer or a manual Wheaton tissue grinder. For cell suspensions, to avoid cell clumping that causes lower RNA yields, cell pellets need to be broken up and resuspended in a small volume of medium before adding the Trizol solution. About 0.2ml of chloroform was then added to the homogenized tissue, mixed and centrifuged at 10,000g for 5 min. The top aqueous phase was then transferred to a 1.5ml-eppendorf tube, then incubated with 0.5ml of 2-propanol at room temperature for 10 min, and centrifuged for 10 min. The RNA pellet was then briefly dried at room

temperature. For cell suspensions, this RNA pellet was ready for subsequent analysis and could be resuspended in an appropriate volume of 0.1mM EDTA/DEPC water. For tissues, subsequent purification steps were necessary to obtain high quality of RNA. Briefly, the dried RNA pellet from the above step was then completely re-dissolved in approximate 0.4 ml of 0.1mM EDTA/DEPC water. Brief incubation of the RNA solution at 50^{0}C was helpful to dissolve the RNA pellet. An equal volume (0.4 ml) of Tris-saturated phenol (pH 4.3)/chloroform mixture (1:1) was then added, mixed and centrifuged for 4 min. The top aqueous phase was then transferred to another 1.5ml eppendorf tube and mixed with a one-tenth volume of 5M LiCl and 1ml of 100% ethanol. After incubating the mixture at -20^{0}C for 30 min, RNA was precipitated by centrifugation at 10,000g for 30 min. The RNA pellet was then washed with 75% ethanol and re-dissolved in a small volume of 0.1mM EDTA/DEPC water. The integrity of isolated RNA was checked by gel electrophoresis using 1% agarose gel. Generally, RNA quality was determined by the presence of two sharp bands of 28S and 18S ribosomal RNAs.

In situ hybridization and combined _in situ_ hybridization/immunohistochemistry

This non-radioactive digoxygenin-based method for *in situ* hybridization on frozen tissue sections is a modified, combined version of protocols from many different laboratories that are publicly available on the internet. Several important observations for

generating RNA anti-sense probes and critical parameters necessary for a good in situ hybridization result are discussed here.

Important considerations for the generation of digoxygenin-labelled anti-sense riboprobes.

To obtain optimal *in situ* hybridization, the length of the anti-sense RNA probe needs to be experimentally determined. For detecting mRNAs of most lymphoid tissue chemokines, I found that a 1-1.5 kb RNA probe usually gives good hybridization signals. To obtain good RNA products from the *in vitro* transcription, a cDNA template needs to be free of polyA sequence. Finally, a cDNA template linearized with a restriction enzyme that leaves 5'-overhang or blunt-end usually generates better RNA product than 3'-overhang cutters. It is necessary to confirm the quality of the RNA product by gel electropheresis before proceeding further. The RNA product obtained from the *in vitro* transcription can be used directly for *in situ* hybridization without further purification or digestion of the DNA template.

Pre-treatment and hybridization of sections.

OCT compound-embeded frozen tissues were sectioned and collected on RNAse-free slides. Sections on slides must be completely air-dried for more than two hours. Slides could be stored at -20^{0}C in dessication for later use; however, freshly cut slides generally give better results than stored slides. After air-drying, slides were warmed up to 50^{0}C for 5 min, then fixed in 4% paraformaldehyde (made in DEPC-phosphate

buffered saline [PBS]) at room temperature for 20 min. Tissue culture-grade PBS is also suitable for this purpose. Slides were then washed twice in PBS and treated with a freshly made solution of 0.1M RNAse-free triethanolamine-HCl pH 8.0 and 0.25% (v/v) acetic anhydride (Sigma) for 10 min, followed by three washes in PBS. Next, slides were placed in a humid chamber with 50% formamide and pre-hybridized in a hybridization solution (50% formamide, 5X SSC, 250µg/ml yeast RNA, 100µg/ml Heparin, 1X Denhardt's, 0.1% Tween-20, 0.1% CHAPS and 5mM EDTA) at 60^{0}C for 1-2 hours. To minimize evaporation, a strip of parafilm was placed on the top surface of the slides. The pre-hybridizing solution was then removed with a pipet withdrawing the solution at the edge of the parafilm strip, replaced with approximately 1µg/ml of probe in a fresh hybridizing solution, and incubated for a further 12-16 hours. The optimal concentration of probe was usually empirically determined.

Washing Steps

After hybridization, it was not necessary to use RNAse-free buffers. Washing slides was done as follows. Slides were put in a trough with a stir bar and washed in 1x SSC at 60°C for 10 min, then in 1.5x SSC at 60°C for 10 min, and in 2x SSC at 37°C for 20 min each. To digest the unhybridized probe, slides were treated with 0.2µg/ml RNAse A in 2x SSC at 37°C for 30 min, then rinsed in 2x SSC at room temperature for 10 min, and washed again twice in 0.2x SSC at 60°C for 30 min each. Slides were then equilibrated in B1 buffer (0.1M maleic acid and 0.15M NaCl, pH 7.5) and incubated in

B2 blocking buffer (B1 buffer containing 1% blocking reagent [Boehringer Mannheim] and 1% normal mouse serum) for one hour at room temperature. For some probes, the longer incubation seemed to cut down the background.

Antibody visualization of digoxygenin

After blocking, an alkaline phosphatase-coupled anti-digoxygenin antibody was added to slides at a dilution of 1:2000 (alternatively, we used unconjugated sheep antibody anti-digoxygenin at 1:1000 dilution followed by alkaline phosphatase-conjugated donkey anti-sheep IgG at 1:200 dilution) in B2 buffer for 1 hour at room temperature. Slides were then washed twice in B1 buffer at room temperature for 10 min each and then rinsed in alkaline phosphatase buffer (1M Tris pH 9.5, 1M $MgCl_2$, 5M NaCl, and 0.1% Tween-20) at room temperature for 5 min. The colored substrate from the enzymatic reaction was developed by adding 4.5µl of NBT and 3.5µl of BCIP for every ml of alkaline phosphatase buffer, and incubated in the dark for 2-20 hours. When the signal reached the desired intensity, the reaction was stopped by submerging the slides in B1 buffer. Slides could be mounted or proceeded to immunohistochemical staining.

Combined immunohistochemistry

Standard immunohistochemistry was done in combination with the above *in situ* hybridization steps by staining with the desired marker after the *in situ* hybridization signals have been completely developed. However, in our hands, we could only

successfully stain for a few markers including B220, peanut agglutinin, and PNad, which seem to be resistant to paraformaldehyde fixation. In general, whether an antibody is suitable for this combined technique needs to be experimentally tested.

Cell staining with the LTβR-Ig fusion protein:

Important considerations:

The LTα1β2 heterotrimer is very unstable, especially at alkaline pH [174], thus care should be taken to minimize any pH increase of the medium. Helpful practices to minimize pH increase in the medium include using large volumes of the medium to resuspend cells (preferably 5-10ml), minimizing air contact with the medium by keeping the vessels closed, keeping the cell suspension on ice, and quickly proceeding to staining (within 4-5 hours after isolating cells from tissues). Decreased detection of surface LTα1β2 was often observed if cells were kept on ice for over 5 hours.

Staining procedure:

Approximately 0.5 to 1 x 10^6 cells were quickly spun down and resuspended in an ice-cold staining buffer (PBS solution containing 2% newborn calf serum and 0.1% sodium azide). To block Fc-receptor non-specific binding, anti-FcR (CD16/CD32, Pharmingen) was added at 1:100 for 10 min. Surface LTα1β2 was detected by adding LTβR-Ig to a concentration of 10μg/ml, followed by anti-human IgG-PE (Jackson

ImmunoResearch, cat#109-116-098) at 1:100 for 20 min each. Mouse or rat IgG binding cross-reactivity of the anti-human IgG-PE reagent were pre-adsorbed by incubation with mouse and rat serum (1:25 dilution or 4% each) for 15 min. Cells were then stained with appropriate antibody combinations for identifying lymphocyte subsets. After each staining step, cells were washed twice with the staining buffer before adding the next antibody. To control for LTα1β2-specific binding of the LTβR-Ig reagent, anti-LTβ pre-blocking was used by incubation with 10 μg/ml BBF6 [102] for 20min. Stained cells could be immediately analyzed by flow cytometry or could be fixed in 4% paraformaldehyde/PBS solution for later analysis.

Generation of membrane-LTα transgenic mice

A DNA fragment that encodes the membrane and stalk domains of the LTβ gene [33, 175] and corresponds to nucleotides 1-450 of the GenBank file U16985 was generated by RT-PCR using first strand cDNA synthesized from splenic total RNAs. A DNA fragment that corresponds to nucleotides 124-609 (GenBank file M16819) and encodes for the receptor-binding domain of the LTα gene [176] was similarly generated. The PCR reaction for the LTβ gene contained 2.5 units of pfu enzyme (Stratagene), 0.1mM dNTPs, 1x pfu buffer and the primers 5'-TAGTAGGGATCCGATATCCTGGATGGGGACACGGGGACT-3' (with BamHI and

EcoRV sites) and 5'-TAGTAGGTCGACATCATCATCATCTTTGTAATCGTTGAGG-3' (with SalI site and a prolactin/Flag sequence encoding the flag epitope DYKDDDD). The PCR amplification conditions were 45 cycles, at 94^0 for 40 seconds, 68^0 for 50 seconds, and 72^0 for 50 seconds. Identical PCR reaction conditions were used for the LTα gene with the following primers 5'-TAGTAGGTCGACCATGGCATCCTGAAACCTGCT-3' (with SalI site) and 5'-TAGTAGATCGATGCGGCCGCCTACAGTGCAAAGGCTCC-3' (with NotI and ClaI sites). The PCR fragments were digested with appropriate restriction enzymes according to the restriction sites designed for the primers and cloned into the pEF-BOS-XC [177]. An EcoRV-NotI fragment containing the LTβ-LTα gene was then removed from the resulting vector and cloned into the end-blunted ClaI site and NotI site of the Ig *kappa*-promoter/enhancer-, late-SV40-polyA-containing pBXD1 vector (obtained from R. Cormall, Oxford University, Headington, UK). The final vector was cut with BamHI and EcoRI to release the transgenic construct for oocyte microinjection. A linearized Ig *kappa* promoter-chimeric membrane-LTβ/LTα minigene that contained the late SV40 polyadenylation site and the Ig *kappa* intronic enhancer and 3' enhancer was microinjected into fertilized C57BL/6J × DBA/2J oocytes according to standard techniques [178]. To screen for transgenic mice, ear DNA was prepared and the transgene was detected by PCR using the above indicated primer pairs corresponding to

the LTβ gene. Founders were then crossed with LTα-deficient mice to generate LTαtg/LTα$^{-/-}$ mice. To distinguish LTα$^{-/-}$ mice from LTα$^{+/-}$ mice, the following primers were used: 5'-TAGTAGGTCGACATGACACTGCTCGGCCGTCT-3', 5'-GCTTGCCGAATATCATGGTGG -3', and 5'-GGACAGAAGAGAGTGGAGAGG -3', which generated two distinct PCR products corresponding to the LTα-targeted allele and the LTα wild-type allele. The PCR amplification conditions were 40 cycles, at 94^0 for 30 seconds, 55^0 for 30 seconds, and 72^0 for 60 seconds. The transgene was heterozygous in all of the transgenic mice examined.

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