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BLC Expression in Pancreatic Islets Causes B Cell Recruitment and Lymphotoxin-Dependent Lymphoid Neogenesis

Sanjiv A. Luther,* Theresa Lopez,† Wei Bai,*§ Douglas Hanahan,† and Jason G. Cyster*‡
*Department of Microbiology and Immunology
†Department of Biochemistry and Biophysics, Hormone Research Institute
University of California, San Francisco
San Francisco, California 94143

Summary

CXCR5, the receptor for B lymphocyte chemoattractant (BLC), is required for normal development of Peyer's patches, inguinal lymph nodes, and splenic follicles. To test the in vivo activity of BLC in isolation of other lymphoid organizers, transgenic mice were generated expressing BLC in the pancreatic islets. In addition to attracting B cells, BLC expression led to development of lymph node–like structures that contained B and T cell zones, high endothelial venules, stromal cells, and the chemokine SLC. Development of these features was strongly dependent on B lymphocytes and on lymphotoxin α1β2 and could be reversed by blocking lymphotoxin α1β2. These findings establish that BLC is sufficient to activate a pathway of events leading to formation of organized lymphoid tissue.

Introduction

Recent studies have identified several chemokines that are constitutively expressed in lymphoid organs (Yoshie et al., 1997; Zlotnik et al., 1999). These "lymphoid" chemokines act as major cues for homeostatic trafficking within lymphoid tissues (Cyster, 1999). The chemokine B lymphocyte chemoattractant (BLC) (also called BCA-1) is produced constitutively by stromal cells in lymphoid follicles and in vitro is an efficacious attractant of naive B cells (Gunn et al., 1998; Legler et al., 1998). In addition, BLC attracts some activated and memory T cells (Ansel et al., 1999), while it shows no effect on naive T cells, peritoneal macrophages, or granulocytes. An important role for BLC in vivo is indicated by the defective trafficking to lymphoid follicles of mature B cells deficient in the BLC receptor CXCR5 (formerly BLR-1) (Förster et al., 1996). Splenic follicles fail to form in CXCR5$^{-/-}$ mice, and instead, B cells organize in ring-like structures around the T zone. Interestingly, CXCR5-deficient animals lack inguinal lymph nodes as well as most Peyer's patches, suggesting an additional role of CXCR5 and BLC in development of certain lymphoid organs (Förster et al., 1996). In addition to its role as a lymphoid-tissue chemokine, BLC has been observed in *Helicobacter pylori*–induced gastric mucosa-associated lymphoid structures and B cell lymphomas (Mazzucchelli et al., 1999). Two other constitutively expressed chemokines, SLC/6Ckine/TCA4/Exodus2 and ELC/MIP3β, are made by cells in the T cell zone and are ligands for CCR7 (Yoshie et al., 1997; Cyster, 1999; Zlotnik et al., 1999). Genetic studies have established an important role for these molecules in dendritic cell and T cell trafficking to T cell areas of secondary lymphoid organs (Förster et al., 1999; Gunn et al., 1999). In contrast to CXCR5$^{-/-}$ mice, CCR7$^{-/-}$ mice develop all Peyer's patches and lymph nodes (Förster et al., 1999).

Parallel studies have established a critical role for tumor necrosis factor (TNF) and lymphotoxin (LT) in development and maintenance of lymphoid architecture (reviewed in Fu and Chaplin, 1999). Mice deficient in LTα, which lack both the soluble LTα3 homotrimer as well as the membrane LTα1β2 heterotrimer, show gross morphological changes, including the absence of Peyer's patches and all lymph nodes (Fu and Chaplin, 1999). LTβ$^{-/-}$ animals have similar defects, although some mucosal lymph nodes are retained (Fu and Chaplin, 1999). Within the spleen of LTα- or LTβ-deficient animals, white pulp cords are severely disorganized, and there is minimal development of stromal cells or expression of BLC, SLC, or ELC (Fu and Chaplin, 1999; Ngo et al., 1999). Receptor-blocking studies have established a constitutive requirement for LTβ receptor (LTβR) signaling to maintain follicular dendritic cell (FDC) networks and high BLC and SLC expression (Mackay and Browning, 1998; Ngo et al., 1999). The more broadly expressed cytokine, TNFα, and its receptor, TNFR1, are also needed for normal development of stromal cells and expression of chemokines in B and T cell areas (Fu and Chaplin, 1999; Ngo et al., 1999).

The lack of certain lymphoid organs in LTα-, LTβ-, and CXCR5-deficient mice as well as the similar disruption of B cell follicles in TNF- and CXCR5-deficient animals suggests that LTα1β2, TNF, and CXCR5 act in a common pathway of lymphoid neogenesis. Recently, B lymphocytes have been recognized as an essential source of LTα1β2 and TNF in the development of follicles and FDC networks (Fu et al., 1998; Gonzalez et al., 1998; Endres et al., 1999). The strong B cell–attracting activity of BLC together with the requirement of B cells as a source of LTα1β2 and TNF suggests that these cytokines may function downstream of BLC in follicle development. However, because LTα1β2 and TNF are required for normal BLC expression (Ngo et al., 1999), it has so far not been possible to test whether these cytokines function downstream. B cells have also recently been found to be important for normal development of Peyer's patches (Golovkina et al., 1999), although it is not yet known whether they are required here as a source of LTα1β2 or TNF.

To study whether BLC is sufficient in vivo to mediate lymphocyte recruitment and possibly to lead to downstream events associated with lymphoid organ development, we generated transgenic mice expressing BLC in β cells of the pancreatic islets. BLC expression was found to be sufficient not only to mediate recruitment

‡To whom correspondence should be addressed (e-mail: cyster@itsa.ucsf.edu).
§Present address: Genentech Inc., 1 DNA Way, South San Francisco, California 94080.

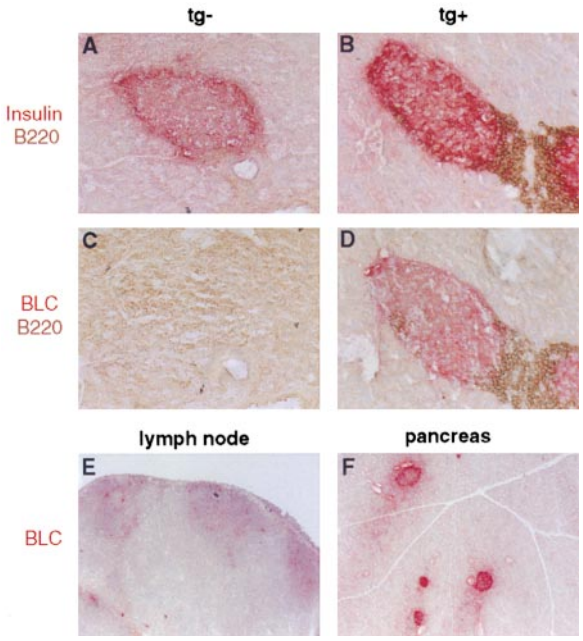

Figure 1. Islet Expression of BLC Protein in RIP-BLC Transgenic Mice Leads to Local B Cell Accumulation

(A–D) Cryosections from pancreas of 8-week-old nontransgenic littermate control mice (tg−; A and C) or RIP-BLC1 transgenic mice (tg+; B and D) were immunohistochemically stained for insulin (A and B) or BLC (C and D) in red and for B lymphocytes in brown (B220; A–D).

(E and F) A cryosection containing both peripheral lymph node (E) and noninfiltrated part of pancreas (F) from a single RIP-BLC1 transgenic mouse was stained to detect BLC. Counterstain with hematoxylin (blue) shows the B cell follicles of the lymph node (E).

Magnifications: 200× (A–D); 50× (E–F).

of large numbers of B lymphocytes but also to cause development of lymph node–like structures. These infiltrates were strongly dependent on B lymphocytes and the cytokine LTα1β2 in order to develop and to be maintained. These findings establish that BLC can function upstream of LTα1β2 in a pathway of lymphoid neogenesis.

Results

Ectopic BLC Expression Mediates B Cell Recruitment

Three lines of transgenic mice were generated that carry the BLC cDNA under control of the rat insulin promotor (RIP). Staining of adjacent histological sections for insulin and BLC revealed that all the islets in transgenic animals were expressing BLC (Figures 1B and 1D), whereas no BLC was detectable in islets of nontransgenic littermates (Figures 1A and 1C). No BLC expression was found in kidney or liver of the transgenic animals (data not shown). By comparing the intensity of immunohistochemical staining in pancreas with staining in lymph nodes, the pancreatic β cells were estimated to be producing several-fold higher amounts of BLC than follicular stromal cells (Figures 1E and 1F).

To determine whether ectopic expression of BLC was sufficient to promote lymphocyte recruitment, we examined hematoxylin-stained pancreas sections from RIP-BLC transgenic mice and nontransgenic littermates. Approximately half of the islets in RIP-BLC transgenic mice were infiltrated to various degrees with small nucleated cells, whereas no infiltrates were detected in the controls. By immunohistochemistry, many of the infiltrating cells were identified as $B220^+$ B lymphocytes (Figures 1B and 1D). Mice derived from each of the three RIP-BLC transgenic lines showed similar B cell–rich infiltrates, and for further experiments we focussed on one of the lines, RIP-BLC1. To obtain a quantitative measurement of the lymphocytes infiltrating the pancreas, lymphoid cells were isolated from pancreas tissue by collagenase digestion and density gradient separation. Flow cytometric analysis revealed many lymphocytes in the isolates from transgenic pancreas, with an enrichment for B lymphocytes compared to peripheral blood (Figures 2A and 2B). By contrast, very few lymphocytes were obtained from nontransgenic pancreas (Figures 2A and 2B). On average, 90% of pancreas-infiltrating lymphocytes in RIP-BLC1 transgenic mice were $B220^+$ B cells, independent of mouse age. The B cells predominantly displayed a naive phenotype: $CD69^-$, IgM^+, $CD19^+$ (data not shown), IgD^+, and $CXCR5^+$ (Figures 3D and 2C). Although B cells predominated, CD4 and CD8 T cells were also present, being increased in number ~10-fold compared to littermate controls (Figures 2A and 2B). Only a small fraction of $CD4^+$ or $CD8^+$ cells showed signs of acute activation (cell size, $CD69^+$; data not shown). To determine whether the T cells could have been directly recruited by BLC, we stained for the BLC receptor CXCR5. CXCR5 is expressed on mature B cells and on some activated and memory T cells but not on naive T cells (Förster et al., 1994; Ansel et al., 1999). The majority of the pancreatic $CD4^+$ and $CD8^+$ T cells expressed CXCR5 (Figure 2C), indicating that many of the T cells may have accumulated in response to BLC. Importantly, however, ~30% of the T cells were $CXCR5^{low/neg}$, suggesting that ectopic BLC expression leads to conditions that allow some T cells to enter the pancreas by a mechanism other than in direct response to BLC.

Ectopic BLC Expression Causes Lymphoid Neogenesis

To further characterize the events that occur downstream of pancreatic BLC expression, large-sized infiltrates in RIP-BLC transgenic mice were examined for the composition and organization of cells. In these infiltrates, the islet architecture was often strongly disturbed, resembling severe insulitis (Figure 3A). Despite the extent of the infiltration, the mice did not show overt signs of diabetes, having normal blood glucose levels and no obvious decrease in the number of β cells, even when 16 months old (data not shown). In all the infiltrates, $B220^+$ B lymphocytes were predominant and localized adjacent to the BLC (and insulin)-producing β cells (Figure 3A). Consistent with the flow cytometric analysis, the large infiltrates contained considerable numbers of $CD3^+$ T cells that localized more distant from the BLC-producing β cells than the B lymphocytes (Figure 3B). $CD11c^+$ dendritic cells were also present,

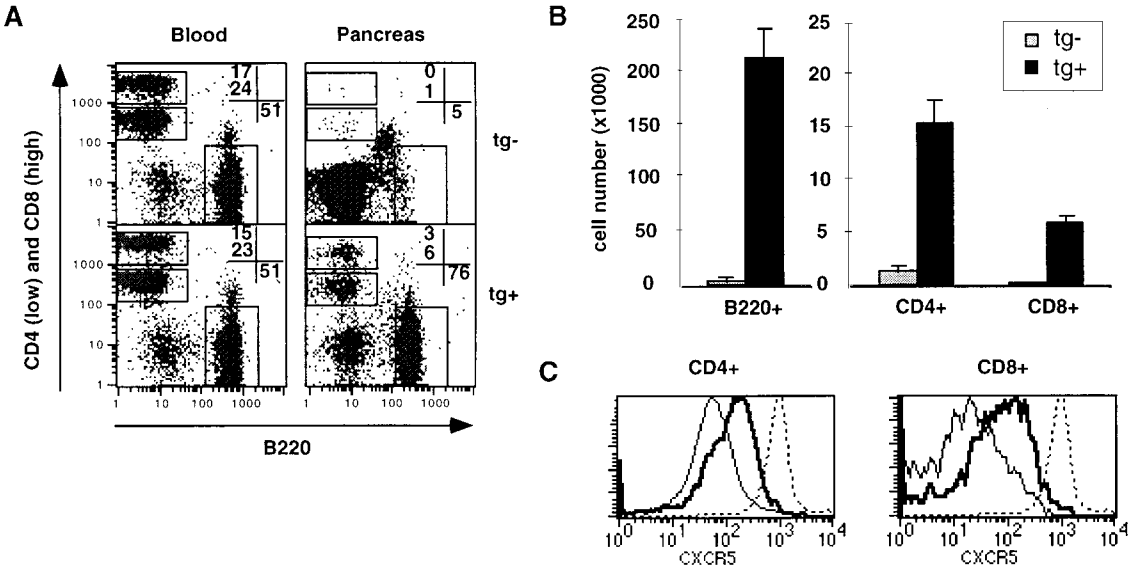

Figure 2. Flow Cytometric Detection of Large Numbers of B Lymphocytes and Smaller Numbers of T Lymphocytes Infiltrating the Pancreas of RIP-BLC Mice

(A–C) Lymphocytes were isolated from blood or perfused pancreas of 10- to 14-week-old nontransgenic littermates or RIP-BLC1 transgenic mice and stained with antibodies to B220, CD4, CD8 and CXCR5.

(A) Representative dot plot analysis of the blood and pancreatic infiltrate (pancreas) of one nontransgenic (tg−) and one transgenic (tg+) mouse. Numbers in the top right of each profile indicate the percentage of total cells within the corresponding gate. CD4 and CD8 were stained with the same fluorophore and are identified by their low (CD4) or high (CD8) fluorescence intensity.

(B) Numbers of $B220^+$, $CD4^+$, and $CD8^+$ lymphocytes isolated from pancreatic infiltrates of littermate controls (tg−) and RIP-BLC transgenic (tg+) mice (mean ± SD; four mice in each group).

(C) Representative histogram showing CXCR5 expression levels on $CD4^+$ and $CD8^+$ lymphocytes isolated from transgenic pancreas (thick line) or similarly processed lymph node (thin line) from the same animal. As a comparison, CXCR5 levels on $B220^+$ B lymphocytes isolated from the same transgenic pancreas are shown (dotted line).

and in most cases the T cells and dendritic cells colocalized and clearly separated from the B cells (Figures 3B and 3C).

The similarity between the organization of the infiltrate and the normal compartmentalization of cells in secondary lymphoid organs prompted us to investigate other features typical for lymphoid structures. An antibody specific for peripheral lymph node addressin, PNAd, stained a considerable number of blood vessels within the lymphocytic infiltrates in RIP-BLC transgenic pancreas (Figure 3D). A subset of these vessels coexpressed the mucosal addressin, MAdCAM (Figure 3E and data not shown). More careful morphological analysis revealed that many of these vessels had thicker walls than other surrounding vessels, suggesting that they had acquired the properties of lymph node HEVs. A few blood vessels also expressed VCAM, an adhesion molecule typically found on activated endothelium (Figure 3G). Only a few scattered $Mac\text{-}1^+$, $MOMA\text{-}1^+$, or large MHC II^+ macrophages were found (data not shown). Staining for BP3, an antigen common to stromal cells in B and T cell areas of lymphoid organs (McNagny et al., 1991), revealed a dense network of cells extending through most of the infiltrate (Figure 3E). This stromal network was negative for the FDC markers FDC-M1 and CD35 except for occasional staining of vascular structures by FDC-M1 (data not shown). Remarkably, staining for the chemokine SLC was detectable within the infiltrates on a subset of HEVs as well as in a dense network in the T cell–rich area (Figure 3F). This finding is likely to explain the presence of $CXCR5^-$ T cells (Figure 2C) and dendritic cells (Figure 3C) in the infiltrates.

To test whether the BLC-induced lymphoid structures formed part of the recirculation pathway of naive lymphocytes, CFSE-labeled splenocytes from anti-hen egg lysozyme (HEL) Ig-transgenic mice were intravenously transferred into RIP-BLC1 transgenic mice. After 12–15 hr the pancreas was removed and analyzed by immunofluorescence. CFSE-labeled cells were found throughout the pancreatic infiltrates of the RIP-BLC recipient mice (Figure 3H) in a frequency similar to peripheral lymph nodes (Figure 3I). To establish whether newly arriving lymphocytes would segregate into their respective T and B cell zones, staining with HEL was used to distinguish the transferred B and T cells. Interestingly, T/B segregation occurred as efficiently in the pancreatic infiltrates as in the peripheral lymph node (Figures 3H and 3I). Together, these findings indicate that expression of BLC is sufficient to induce many features associated with secondary lymphoid organs, including blood vessel differentiation, vascular addressin upregulation, stromal cell differentiation, chemokine induction, T/B segregation, and efficient recirculation of naive lymphocytes.

BLC-Induced Lymphoid Neogenesis Is B Cell Dependent

Although all the islets in transgenic animals express BLC, development of HEV structures and stromal cell networks was only observed in islets containing a

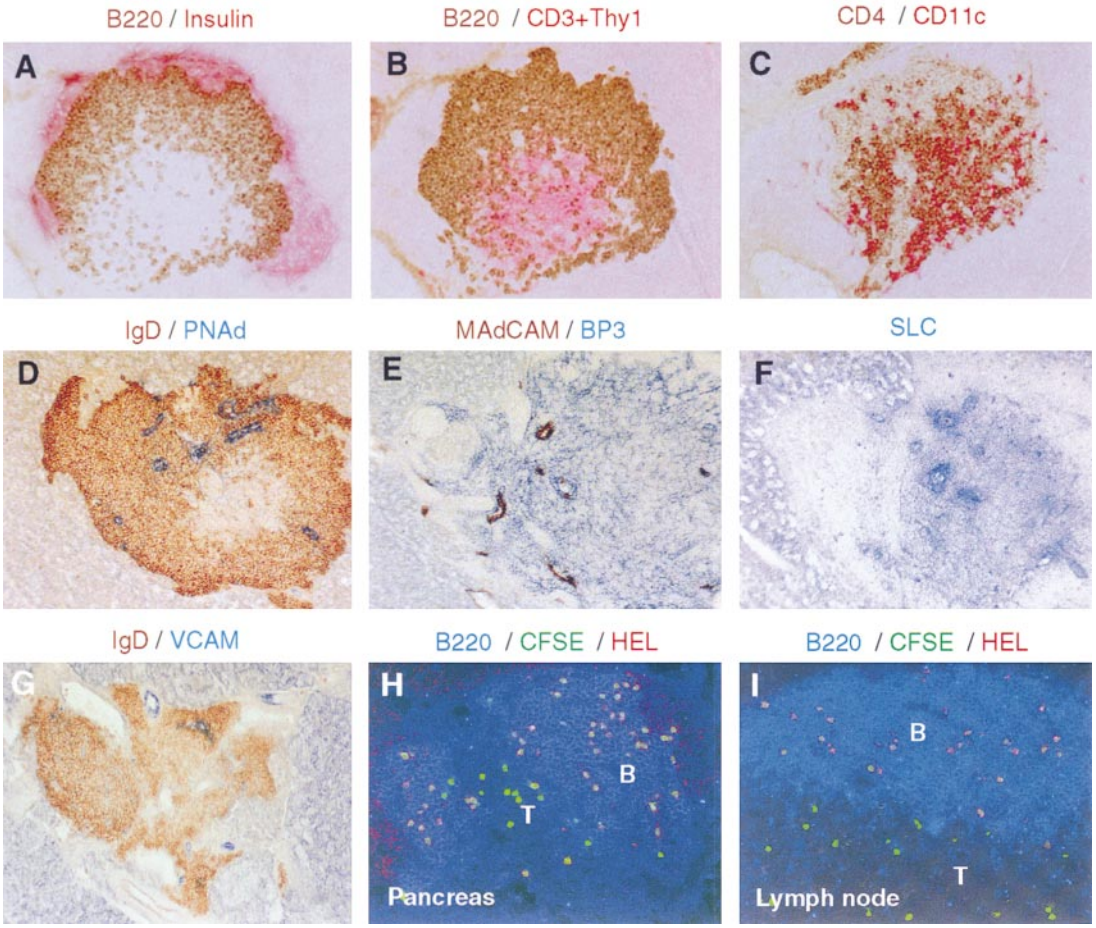

Figure 3. Large Infiltrates in RIP-BLC Transgenic Pancreas Show Many Characteristics of Lymph Nodes

(A–F) Immunohistochemical analysis on serial sections (A–C and D–F) of RIP-BLC1 transgenic pancreas. Sections were stained for the markers indicated, and the color of the label corresponds to the color of the immunohistochemical reaction product. Examples shown are representative of more than 50 large infiltrates examined.

(G) Example of VCAM expression in another large infiltrate. Note that the blue background staining in the exocrine tissue in some sections was also seen in nontransgenic controls and is nonspecific.

(H and I) Immunofluorescence microscopy on pancreas (H) and lymph node (I) of a RIP-BLC1 transgenic mouse 14 hr after intravenous injection of CFSE-labeled anti-HEL Ig-transgenic splenocytes. Transferred T lymphocytes show green cytoplasmic (CFSE) staining and can be distinguished from transferred HEL-specific B lymphocytes that show both green cytoplasmic staining and red surface staining for HEL antigen (H and I). Red background staining outside of infiltrate is nonspecific (H).

T, T cell–rich zone; B, B cell–rich zone (follicle). Magnifications: 145× (A–C); 100× (D–I).

lymphocytic infiltrate. These observations suggested that BLC was not able to directly induce development of HEVs or stromal cells and that these effects were secondary to B lymphocyte recruitment. This possibility was tested by crossing the RIP-BLC1 transgene onto a B cell–deficient ($\mu M^{-/-}$) background (Kitamura et al., 1991). To score the effect of B cell deficiency, we first measured the frequency and size of pancreatic infiltrates in a group of wild-type ($\mu M^{+/+}$ and $\mu M^{+/-}$) RIP-BLC transgenic mice. Three stages of infiltrate composition were defined and will be described as small, medium, and large infiltrates. Small infiltrates (<30 cells/infiltrate in a section) were made up mainly of B cells with very few T cells or dendritic cells and lacked features such as PNAd or $MAdCAM^+$ HEVs, $BP3^+$ stromal cells, or SLC expression (see Figure 1B). Medium-sized infiltrates (between 30 and 300 cells) were characterized by an increased number of B cells, T cells, and dendritic cells as well as HEVs but little or no BP3 and SLC expression and varying T/B segregation. Finally, large-sized infiltrates (more than ~300 cells; see Figure 3) showed well defined T and B cell–rich zones along with extensive BP3 and SLC expression. In wild-type RIP-BLC1 transgenic mice, 5%–15% of the infiltrates were large, 40%–50% medium, and 40%–50% small (Figure 4B).

In B cell–deficient ($\mu M^{-/-}$) RIP-BLC1 mice, very few infiltrated islets were observed (Figures 4A, 4D, and 4E). Although the infiltrates in wild-type RIP-BLC1 mice are B cell dominated (Figure 4C), the majority of infiltrates also contained T cells (Figures 4A and 4C). From these observations, we estimate there was a 10-fold decrease in the number of T cell–containing infiltrates in mice lacking B cells (Figure 4A). An analysis of a large number of hematoxylin-stained sections revealed that despite

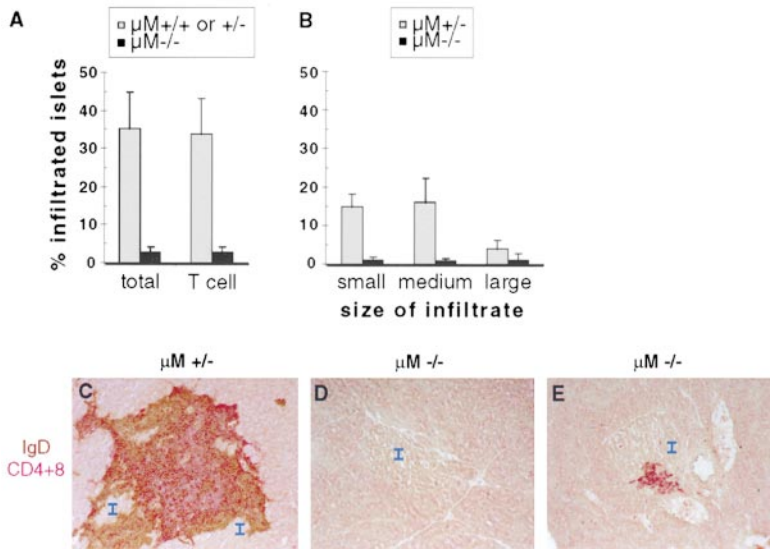

Figure 4. Lymphoid Neogenesis in RIP-BLC Pancreas Is Largely B Cell Dependent

RIP-BLC1 transgenic mice on the $\mu M^{-/-}$ background (B cell deficient) were analyzed for the frequency (A), size (B), and histological appearance (C–E) of the islet-associated infiltrates.

(A and B) More than 350 islets of 3 transgenic $\mu M^{+/-}$ and $\mu M^{-/-}$ littermates were stained with hematoxylin and scored for the presence of mononuclear cells associated with pancreatic islets (A, "total") and for the size of the infiltrate (B; see Experimental Procedures for details). In (A, "T cell") the frequency of islets containing T cells was scored in $\mu M^{+/+}$, $\mu M^{+/-}$, and $\mu M^{-/-}$ mice by staining for CD4/8 (minimally 3 T cells per islet). More than 70 islets were analyzed.

(C–E) Representative immunohistochemical staining of islets in RIP-BLC1 transgenic animals on the $\mu M^{+/-}$ (C) or $\mu M^{-/-}$ (D and E) background.

I, islet of Langerhans. Magnification: 100×.

their low frequency, some infiltrates in the B cell–deficient mice were able to progress to medium and large sizes (Figure 4B). Closer analysis showed that these infiltrates were composed mainly of $CD4^+$ cells, with fewer $CD8^+$ and $CD11c^+$ cells (Figure 4E and data not shown). A single very large infiltrate was observed amongst 350 islets examined, and this infiltrate contained HEVs ($PNAd^+$, $MAdCAM^+$) and an extensive stromal cell network (data not shown). Overall, these results indicate that B cells and signals given by B cells set up the conditions for other cell types to immigrate or differentiate.

BLC-Induced Lymphoid Neogenesis Is LTα Dependent

To explore the possible signals that are provided by B lymphocytes, RIP-BLC1 mice were crossed onto $TNFR1^{-/-}$ and $LT\alpha^{-/-}$ backgrounds. Analysis of $TNFR1^{-/-}$ RIP-BLC1 transgenic mice for the frequency and size of pancreatic infiltrates showed no differences compared to wild-type RIP-BLC1 littermates (Figure 5A), indicating that TNFR1 is not essential for the formation of BLC-induced lymphoid structures. Similar analysis of $LT\alpha^{-/-}$ RIP-BLC1 mice revealed that there was no change in the total frequency of BLC-induced infiltrates, but the average size of the infiltrates in $LT\alpha^{-/-}$ mice was markedly reduced, despite BLC levels comparable to wild-type mice (Figure 5B and data not shown). No large infiltrates were detected in over 350 islets examined, and the number of medium infiltrates was 50% less. Comparing examples of the largest infiltrates present in $LT\alpha^{-/-}$ RIP-BLC1 mice with those in wild-type RIP-BLC1 mice revealed not only the marked difference in size (Figures 6A and 6B) but also the deficiency in accumulation of T cells and dendritic cells (data not shown). In addition, there was a strong reduction in fully developed $PNAd^+$ HEVs (Figures 6A and 6B), stromal cell networks, and SLC expression (data not shown). The most developed structures in LTα-deficient mice were similar in composition to size-matched structures in wild-type mice, consisting mainly of IgD^+ B cells (see Figure 1B). Together, these results suggest that $LT\alpha1\beta2$–LTβR interactions are not essential for the initial B cell infiltration but are required in a subsequent, potentially B cell–mediated step that leads to HEV development, chemokine expression, and more extensive infiltration or retention of T and B cells.

Maintenance of BLC-Induced Lymphoid Structures Depends on LTβR Signaling

The analysis of $LT\alpha^{-/-}$ and $TNFR1^{-/-}$ RIP-BLC mice suggested a developmental role for the $LT\alpha1\beta2$ heterotrimer in this model system. In order to test whether $LT\alpha1\beta2$ was also required for the maintenance of established islet-associated lymphoid structures, RIP-BLC1 transgenic mice were treated for 2 weeks with LTβR-Ig fusion protein to inhibit signaling via LTβR. Additional animals were treated with TNFR1-Ig or, as a negative control, with human LFA3-Ig. Treatment with the TNFR1-Ig had no effect on the frequency or size of the infiltrates (Figure 5C), consistent with the findings in TNFR1-deficient mice (Figure 5A). By contrast, treatment with LTβR-Ig led to the disappearance of the large-sized infiltrates and to a partial reduction in the size and number of the medium-sized infiltrates (Figure 5C), similar to $LT\alpha^{-/-}$ RIP-BLC1 mice (Figure 5B). Immunohistochemical analysis of the largest infiltrates in LTβR-Ig- and LFA3-Ig-treated groups demonstrated that the LTβR-Ig treatment disrupted most of the $PNAd^+$ and $MAdCAM^+$ HEVs (Figures 6D and 6F, compare with 6C and 6E), $BP3^+$ stromal cell networks (Figure 6F versus 6E), and SLC expression within the infiltrates (Figure 6H versus 6G). Interestingly, constitutive SLC expression by lymphatic vessels in the pancreas was not affected by the LTβR-Ig treatment (Figure 6G and 6H, insets), showing the inhibitory effect was specific to the SLC expression induced in the infiltrate. Lymphatic SLC expression also appeared normal in $LT\alpha^{-/-}$ mouse pancreas (data not shown). In summary, treatment with LTβR-Ig fusion protein was able to substantially reverse the BLC-induced infiltration and recapitulate the phenotype observed in $LT\alpha^{-/-}$ RIP-BLC1 transgenic mice.

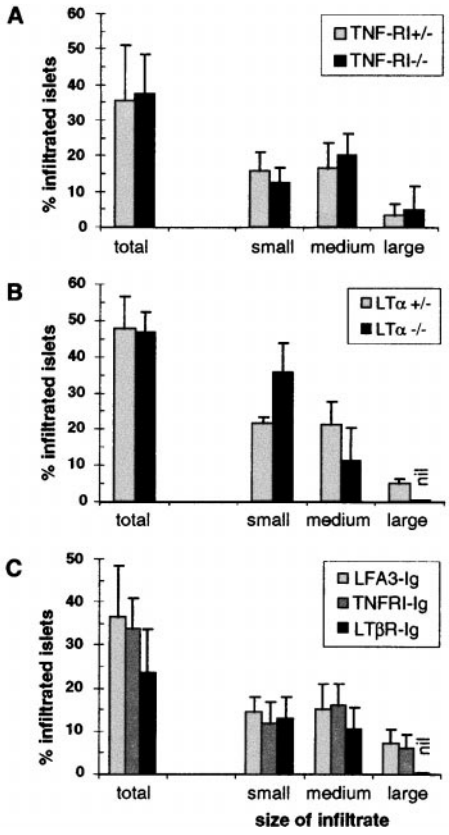

Figure 5. Formation and Maintenance of Large Lymphoid Structures in RIP-BLC Transgenic Pancreas Is Dependent on Signals via LTβR but Not TNFR1

(A and B) Pancreas from RIP-BLC1 trangenic mice crossed onto the TNFR1$^{-/-}$ (A) or LTα$^{-/-}$ (B) background were sectioned and analyzed for the frequency and size of islet-associated, mononuclear infiltrates using hematoxylin staining. Results were derived from more than 350 islets of 3 transgenic mice in each group (7–12 weeks of age).

(C) The identical analysis as in (A) and (B) was performed on RIP-BLC1 transgenic animals that were injected either 3 times (TNFR1-Ig) or 4 times (LFA3-Ig, LTβR-Ig) with 100 μg of soluble fusion protein over 11 or 15 days, respectively. LFA3-Ig served as a negative control. More than 300 islets from 3 animals in each group were analyzed (10–14 weeks of age). Immunohistochemical analysis was performed on spleen and lymph nodes from each of the treated animals, and disruption of FDC markers occurred in TNFR1-Ig- and LTβR-Ig-treated animals as previously observed (Mackay and Browning, 1998; Rennert et al., 1998).

Discussion

The above studies show that expression of the CXCR5 ligand BLC is sufficient to induce lymphoid neogenesis. Development of BLC-induced lymphoid structures in pancreatic islets is strongly dependent on recruitment of B lymphocytes and on the activity of the cytokine LTα1β2. These findings contrast with ectopic expression studies for other chemokines, including MCP1, IL-8, and KC/Gro, where the effects were limited to recruitment of receptor-bearing cells (Lira et al., 1994; Simonet et al., 1994; Fuentes et al., 1995; Nakamura et al., 1995). Our findings with BLC provide a novel example of a chemokine that is sufficient not only to cause cell recruitment but also to induce a program of downstream events (summarized in Figure 7). This pathway is likely to play an important role within BLC-expressing inflammatory lesions (Mazzucchelli et al., 1999). BLC-mediated recruitment of CXCR5-expressing cells that act as a source of LTα1β2 is also likely to be part of the Peyer's patch and inguinal lymph node developmental program (Förster et al., 1996; Nishikawa et al., 1998).

The ectopic lymphoid structures that develop in RIP-BLC transgenic animals are strikingly similar to structures observed previously in RIP-LTα and RIP-TNF transgenic animals (see Kratz et al., 1996). Detailed characterization of the RIP-LTα transgenic mice has shown that large numbers of lymphocytes accumulate within the islets and sort into B and T cell–rich areas (Kratz et al., 1996). VCAM, ICAM1, MAdCAM, and PNAd are upregulated (Kratz et al., 1996; Cuff et al., 1999), and chemokine expression is also increased (Cuff et al., 1999), although expression of BLC and SLC has not yet been analyzed. Based on a combination of in vitro and in vivo findings and on the broad expression pattern of TNFR1, it is thought that LTα3 homotrimer made by the pancreatic β cells directly induces VCAM, MAdCAM, and ICAM1 expression on islet endothelial cells (Ware et al., 1995; Kratz et al., 1996; Sacca et al., 1997; Cuff et al., 1998, 1999). As expected from the lack of expression of LTβ in pancreatic cells, LTα1β2 heterotrimer is not required for many of the events that occur in the RIP-LTα transgenic animals (Sacca et al., 1998). By contrast, in the RIP-BLC1 transgenic mice, the development of infiltrates requires LTα1β2 but does not require TNFR1 and, notably, can be reversed by LTβR-Ig treatment (Figure 7). The similarity of the infiltrates in LTβR-Ig-treated and LTα$^{-/-}$ RIP-BLC1 mice is in accord with previous studies on the involvement of the LTβR-LTα1β2 receptor-ligand pair in lymphoid development (Fu and Chaplin, 1999) and strongly supports the conclusion that the active cytokine is LTα1β2 and not LIGHT, an alternative LTβR ligand (Mauri et al., 1998). These findings fit well with previous studies showing that B lymphocytes are a source of LTα and LTβ (Fu et al., 1998; Gonzalez et al., 1998; Endres et al., 1999), and they suggest a model in which BLC-recruited cells express LTα1β2, and this cytokine then plays a limiting role in inducing downstream events (Figure 7). Consistent with this, the LTβR is expressed by multiple cell types, including endothelial cells and fibroblasts, and it is highly expressed within most organs, including pancreas (Hochman et al., 1996; Degli-Esposti et al., 1997). It is interesting to consider that despite the ability of TNFR1 signaling to induce formation of lymphoid structures (see Sacca et al., 1998), this receptor is not essential for infiltrate formation in the RIP-BLC transgenic animals (see Figure 5). However, it remains to be investigated whether TNF plays a necessary role in infiltrate development when LTα1β2 is absent, accounting for the incomplete inhibition of medium-sized infiltrate formation in LTβR-Ig-treated and LTα$^{-/-}$ RIP-BLC1 transgenic mice (Figure 7). Overall, these observations indicate that within infiltrates dominated by B lymphocytes, LTα1β2 will be functionally more important than TNF or LTα3. Furthermore, they indicate that in addition to the previously described role of LTα1β2 in promoting BLC expression (Ngo et al., 1999), this cytokine is likely to have a role downstream of BLC in the pathway leading to

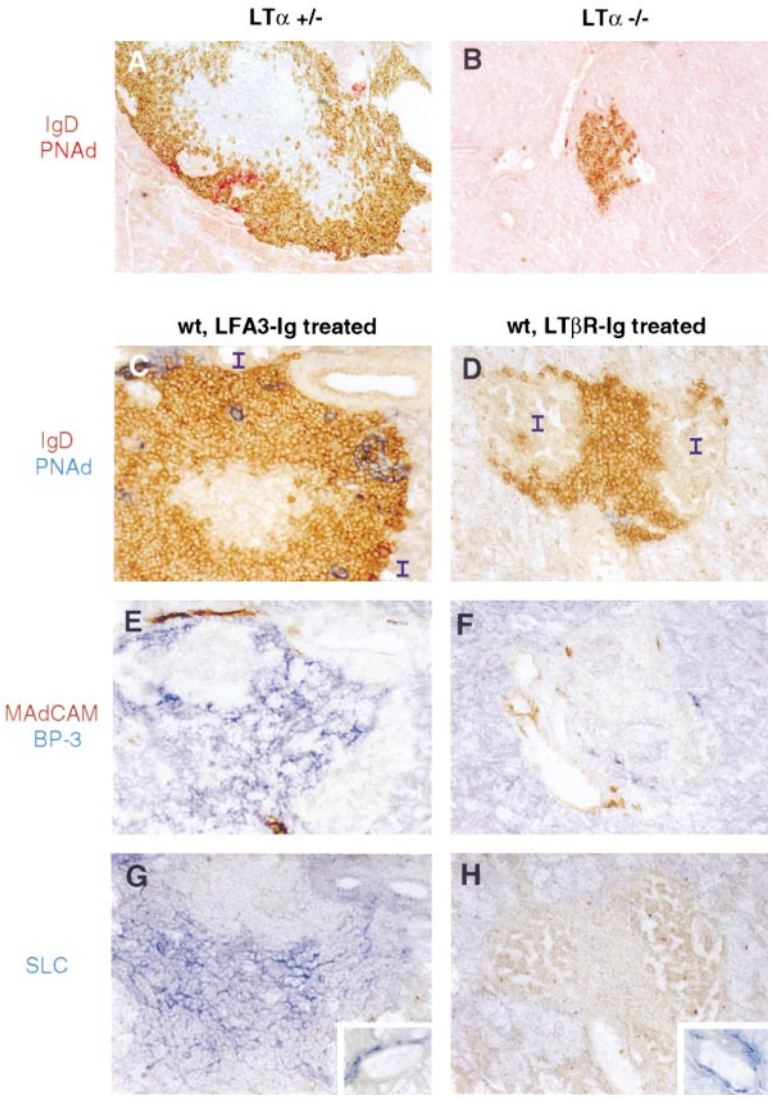

Figure 6. Lymphotoxin $\alpha 1\beta 2$ Is Required Downstream of BLC-Mediated Signals for the Development and Maintenance of HEVs, Stromal Cell Networks, and SLC Expression

(A and B) Immunohistochemical analysis of sections from RIP-BLC1 transgenic littermates on a $LT\alpha^{+/-}$ (A) or $LT\alpha^{-/-}$ (B) background. Sections were stained for IgD (brown) and PNAd (red). Faint blue staining in (A) shows the hematoxylin-positive T cell–rich zone in the infiltrate.

(C–H) Immunohistochemical analysis of sections from RIP-BLC1 transgenic mice treated for 15 days with either LFA3-Ig (C, E, and G, serial sections) or LTβR-Ig (D, F, and H, serial sections) fusion protein. Sections were stained with IgD or MAdCAM in brown and PNAd, BP-3, or SLC in blue, as indicated. One representative example of more than 15 stained infiltrates per group is shown. Insets (in G and H) show transgene-independent SLC expression in lymphatic vessels of both LFA3-Ig- and LTβR-Ig-treated mice. The lymphatic vessels in each case are located adjacent to larger blood vessels that are SLC negative. I, islet of Langerhans; wt, wild-type RIP-BLC1 transgenic mice. Magnification: 100× (A and B); 200× (C–H).

development of CXCR5-dependent lymphoid structures.

Many other cytokines have been ectopically expressed in pancreatic islets, including IFNα, IFNγ, TGFβ, IL-2, IL-4, IL-6, IL-10 (see Mueller et al., 1997), and MCP1 (Grewal et al., 1997). However, none of these transgenic animals appear to develop the organized infiltrates seen in animals expressing LTα, TNF, or BLC. Especially pertinent are the RIP-IL-4 and RIP-IL-6 transgenic animals, which show B cell–rich accumulations but fail to develop organized lymph node–like structures (Campbell et al., 1994; DiCosmo et al., 1994; Mueller et al., 1997), suggesting that B cell accumulation alone is not sufficient to activate lymphoid neogenesis. These cytokines apparently are unable to substitute for the need of the LT/TNF signal and are also not able to induce these cytokines to a sufficient degree. Taken together with our findings, these observations support the notion that cells recruited in response to BLC are a significant source of lymphotoxin. A possibility that we are currently investigating is that BLC directly induces $LT\alpha 1\beta 2$ expression in B lymphocytes.

Development of the infiltrates in RIP-BLC transgenic mice has many similarities to Peyer's patch development. The predominance of B cells and the high level of BLC expression in the RIP-BLC infiltrates is similar to Peyer's patches. Peyer's patch frequency and size is severely reduced in mice lacking either CXCR5 (Förster et al., 1996) or B lymphocytes (Golovkina et al., 1999), and they are totally absent in $LT\alpha 1\beta 2$-deficient mice (Fu and Chaplin, 1999), while being only partially affected by TNF and TNFR1 deficiency (Fu and Chaplin, 1999). $LT\alpha 1\beta 2$ is needed in at least two steps during Peyer's patch organogenesis, with the first step being an inductive one around embryonic day 14 (Nishikawa et al., 1998) and the second one occurring perinatally (Rennert et al., 1996; Yoshida et al., 1999). The late requirement for $LT\alpha 1\beta 2$ is consistent with a role for B cells as a LT source since lymphocytes emerge in the periphery around birth (Rennert et al., 1996). From our studies and the strong requirement for CXCR5 and B cells, it can be proposed that the B cells recruited in response to BLC act as a critical source of lymphotoxin in Peyer's patch development.

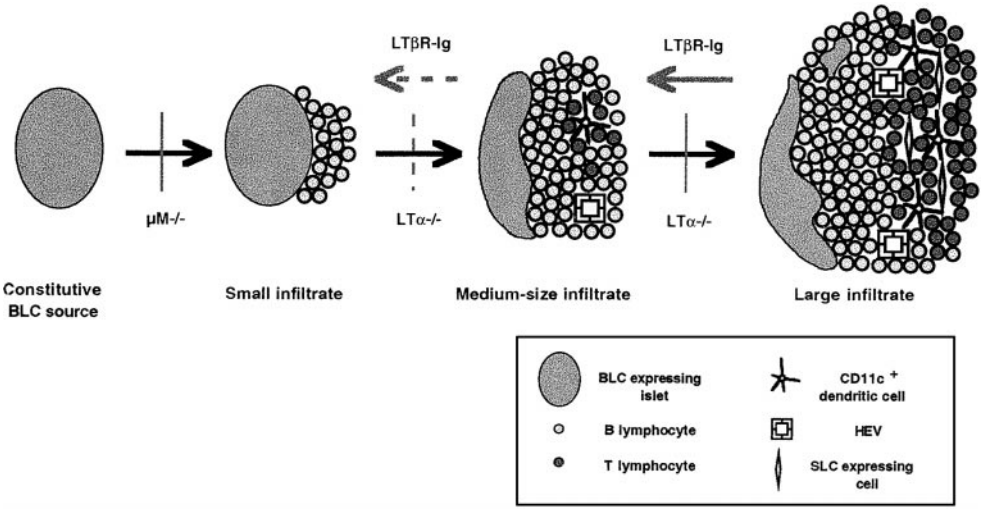

Figure 7. Summary of the Steps and Signals in BLC-Induced Lymphoid Neogenesis

The observations from the histological analysis of infiltrates found in RIP-BLC1 transgenic pancreas were compiled into a simplified model showing the progression from a noninfiltrated islet to a large and highly organized infiltrate that disrupts the BLC-expressing islet of Langerhans (which is a cluster of up to several hundred cells). Plain gray lines indicate a strong requirement, and a dotted gray line indicates a partial requirement for a signal or cell type to progress to the next stage of infiltrate size and organization. For simplicity, stromal cells found in the B cell–rich area of the infiltrate, and macrophages were omitted. In addition to the cell types shown, the progression of infiltrates to medium and large size is accompanied by an upregulation of vascular addressins (PNAd, MAdCAM, and VCAM) on endothelium and by the induction of SLC on endothelium and other cells, presumably stromal cells. These molecules are likely to contribute to the accumulation of naive T cells and dendritic cells in the large infiltrates. It should be noted that while the RIP-BLC1 mice have shown a role for LTα1β2 downstream of BLC, LTα1β2 also plays an important role upstream of BLC (Ngo et al., 1999).

Inguinal lymph nodes also fail to develop in CXCR5-deficient mice (Förster et al., 1996). However, development of these nodes is not affected in B cell–deficient animals, indicating that BLC may act to recruit another CXCR5-expressing cell type that promotes lymphoid organogenesis. Accumulating evidence suggests the involvement of $IL7R^{+}CD4^{+/-}CD3^{-}$ cells in this process (Mebius et al., 1997; Yokota et al., 1999; Yoshida et al., 1999). Like B cells, these cells have been found to express both CXCR5 and lymphotoxin (Mebius et al., 1997; Yoshida et al., 1999). Therefore, perhaps inguinal lymph node development depends on CXCR5/BLC-mediated recruitment of $IL7R^{+}CD4^{+/-}CD3^{-}$ cells, leading to a series of events similar to those observed in the pancreatic infiltrates following B cell recruitment. Since development of all lymph nodes appears to depend on $IL7R^{+}CD4^{+/-}CD3^{-}$ cells (Yokota et al., 1999), BLC/CXCR5 may represent only one of the ligand/receptor pairs involved in recruitment of these cells to sites of lymphoid organ development.

A striking feature of the large infiltrates in RIP-BLC transgenic mice is the separation of lymphocytes into B and T–cell rich areas, with the B cell–rich areas always being found proximal to the BLC-expressing islet cells. Their designation as B and T zones is supported by the rapid migration of transferred B and T cells into the appropriate zones (see Figure 3H). How these compartments become established is unclear, although it seems possible that differences in the efficiency with which B and T cells migrate to the proximity of BLC-producing β cells may help initiate a relative separation of B and T cells. Induction of SLC expression, which occurs selectively in the T cell–rich region (see Figure 3), is then likely to reinforce the compartmentalization. An important implication of these findings is that once the location of BLC production is defined, the position of the T cell–rich compartment is fixed relative to this location. Recently, it has been established that there are at least two genes for SLC, a form predominantly expressed within lymphoid organs and a second that is expressed by lymphatic vessels in nonlymphoid organs (Vassileva et al., 1999). We demonstrate here that expression of SLC in lymphatic endothelium is lymphotoxin independent (see Figure 6), in contrast to the requirement for LTα1β2 for SLC expression in secondary lymphoid tissues (Ngo et al., 1999) and in the RIP-BLC islet infiltrates. Which cell type(s) provides the LTα1β2 required for SLC expression in the infiltrates is under further investigation. Interestingly, although SLC-expressing cells develop in the T cell areas of the islet infiltrate, extensive immunohistochemical analysis failed to reveal development of FDC in the B cell–rich areas. Unfortunately, the large amount of BLC produced by the pancreatic β cells made it impossible to establish whether BLC was expressed by the stromal cells present within the B cell–rich areas. The failure to observe development of FDC made it impossible for us to test whether LTα1β2 is needed downstream of BLC for development of this cell type. FDC development has been observed at sites of chronic autoimmune inflammation as well as within the infiltrates of RIP-LTα transgenic animals (Kratz et al., 1996), and their apparent absence in the RIP-BLC transgenic animals was unexpected. Perhaps the high BLC production by the β cells in RIP-BLC transgenic animals acts in a feedback inhibitory loop to limit FDC development.

The effector activities of B lymphocytes in immune

responses are usually thought to be limited to antigen presentation and production of antibody. However, the finding that B cells are an important source of LTα, LTβ, and TNF for formation of follicles and FDCs (Fu et al., 1998; Gonzalez et al., 1998; Endres et al., 1999) raised the possibility that B cells could have additional influences. We have now shown that lymphoid neogenesis can be initiated as a result of BLC-mediated B cell recruitment to a nonlymphoid tissue. In addition to constitutive expression in the follicles of all secondary lymphoid organs, expression of BLC has been reported in the gastric mucosa–associated lymphoid structures and B cell lymphomas that form in response to *Helicobacter pylori* infection (Mazzucchelli et al., 1999). B cells predominate in the infiltrates associated with several other chronic inflammatory diseases, such as rheumatoid arthritis and thryoiditis, and it will be important to determine whether BLC is induced in these diseases. An important implication of our observations (Figure7) is that compounds designed to inhibit BLC or the key downstream mediator LTα1β2 have the potential to diminish the severity of BLC-expressing inflammatory lesions.

Experimental Procedures

Transgenic Mice

The full-length cDNA of murine BLC (Gunn et al., 1998) was cloned into a ClaI site of the RIP7 promotor construct (Olson et al., 1998). Pronuclear injection of linearized RIP-BLC minigene lacking the vector sequences was performed according to standard techniques into fertilized B6D2F1 oocytes. Three founders with an approximate copy number of 19, 9, and 4 copies were backcrossed to C57BL/6 mice. One line, RIP-BLC1 (19 copies), was backcrossed for 3–7 generations, and mice were used at 6–16 weeks of age. RIP-BLC1 mice were used to cross to $\mu M^{-/-}$ mice (Jackson Laboratories, West Grove, PA) (Kitamura et al., 1991), $LT\alpha^{-/-}$ (Jackson Laboratories) (Togni et al., 1994), or $TNFR1^{-/-}$ mice (Peschon et al., 1998). $\mu M^{-/-}$ and $TNFR1^{-/-}$ mice were on a B6 background, while $LT\alpha^{-/-}$ mice were on a mixed B6 and 129 background. The transgene was detected by PCR using the following primers: 5′-CAACCCTGAC TATCTTCC (RIP7, forward) and 5′-GAGATGGATAGTGGCTTCAGG CAG (BLC, reverse). Some adult RIP-BLC1 mice received 3–4 intraperitoneal injections (every 3 days) of 100 μg soluble human LFA3-Ig, murine LTβR-Ig, or TNFR1-Ig fusion protein as previously described (Rennert et al., 1998). Tissues were harvested 3 days after the last injection.

CFSE Staining and Adoptive Transfer

Splenocytes from anti-HEL Ig-transgenic mice (Goodnow et al., 1988) were resuspended in RPMI with 2% FCS and labeled with 20 μM 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 10 min at 37°C. Cells were washed in RPMI with 15% FCS and injected into the lateral tail vein of RIP-BLC1 mice and tissues harvested after 12–15 hr.

Flow Cytometry

Venous blood was collected in Alsever's solution by tail bleeding or puncturing of the aorta and erythrocytes lysed. Mice were killed using CO_2, their tissues removed and either frozen in OCT compound (Miles, Elkhart, IN) for sectioning or infiltrating cells isolated (Allison et al., 1992) and stained for flow cytometry. For CXCR5 staining, cells were stained and analyzed as previously described (Ansel et al., 1999). In some experiments, mice have been anesthetized using a 1:40 dilution in PBS of 100% Avertin (Sigma-Aldrich) in tertiary amyl alcohol, and then perfused using PBS to reduce blood contamination in pancreas preparations.

Immunohistochemistry and Immunofluorescence Microscopy

For immunohistochemistry, cryostat sections (10 μm) were collected on superfrost plus slides (Fisher Scientific, Pittsburgh, PA), fixed for 10 min in acetone (4°C), dried, and stored at −20°C. Slides were stained, developed, and analyzed as previously described (Ngo et al., 1999), except for the following unconjugated antibodies: sheep anti-IgD (Binding Site, San Diego, CA), rat IgM anti-PNAd (kindly provided by Steve Rosen, University of California San Francisco, CA), rat anti-MAdCAM (PharMingen), rat anti-FDC-M1 (kindly provided by Marie Kosco, Serono, Geneva, Switzerland), goat anti-mouse BLC and SLC (R&D Systems, Minneapolis, MN), and guinea pig anti-insulin (Linco, St. Charles, MO); for biotinylated reagents: hamster anti-CD3, mouse anti-Thy1.2 (Caltag), rat anti-Mac-1, mouse anti-rat IgM (Caltag), hamster anti-CD11c, mouse anti-$I\text{-}A^p$, rat anti-VCAM (PharMingen), donkey anti-goat IgG (Jackson Laboratories), and goat anti–guinea pig IgG (Vector Laboratories, Burlingame, CA); for alkaline phosphatase–coupled reagents: swine anti-goat IgG (Caltag) and streptavidin-ABC (Vector Laboratories); for peroxidase-coupled reagents: rabbit anti-sheep IgG (Jackson Laboratories) and streptavidin (Amersham Pharmacia Biotech); and for an additional substrate for alkaline phosphatase (Fast Blue [Sigma]/Napthol AS-MX). Immunofluorescence microscopy was performed as previously described (Ansel et al., 1999).

Quantification of Immunohistochemistry

Every fifteenth 10 μm section from the pancreas was counterstained with hematoxylin and the number of mononuclear cells per islet counted. Infiltrates were scored as noninfiltrated (<3 mononuclear cells/islet), small (3–30 cells), medium (31–300 cells), and large (>300 cells). This procedure was applied for 80–140 islets per pancreas. During the enumeration procedure, it was observed that some of the large infiltrates appeared in more than one of the 10 μM sections and sometimes in as many as six. Although this may have caused the frequency of large infiltrates to be somewhat overestimated compared to the small- and medium-sized infiltrates, it does not affect comparisons made between groups of mice.

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