Chronic Inflammation Caused by Lymphotoxin Is Lymphoid Neogenesis
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Summary
In presenting a unifying concept for chronic inflammation and lymphoid organogenesis, we suggest that lymphotoxin’s (LT, LT-α, TNF-β) crucial role in these processes is pivotal and similar. Chronic inflammatory lesions that developed in the kidney and pancreas at the sites of transgene expression in rat insulin promoter-LT (RIP-LT) mice resembled lymph nodes with regard to cellular composition (T cells, B cells, plasma cells, and antigen-presenting cells), delineated T and B cell areas, primary and secondary follicles, characteristic morphologic and antigenic (ICAM-1, VCAM-1, MAdCAM-1, and PNAd) features of high endothelial venules, and ability to respond to antigen and undergo Ig class switching when obtained from mice immunized with SRBC. The vascular changes, with the exception of PNAd, appear to be the direct consequence of transgene derived LT expression, as they were also observed in RIP-LT mice lacking mature T and B cells. These data show that LT-induced chronic inflammation has the characteristics of organized lymphoid tissue.

Chronic inflammation, a complex pathophysiologic process characterized by an accumulation of mononuclear cells, is associated with the response to invading pathogens, neoplastic transformation, or autoimmune recognition of self-antigens. This is usually initiated by events involving macrophages and T cells and is perpetuated through recruitment of additional T cells, B cells, and macrophages. The process can lead to elimination of the pathogen or tumor, but frequently results in tissue damage. The cells in the infiltrate influence the severity of clinical signs and, in addition to the elimination of the causative stimulus, can perpetuate the lesion, resulting in exacerbation in diseases such as multiple sclerosis and rheumatoid arthritis. They can also contribute to resolution of the lesion through suppression of inflammatory activity (1). Although the classical definition of chronic inflammation includes tissue destruction and loss of function (2), several instances of chronic inflammation have been described in which cellular infiltration occurs without tissue destruction. These include insulitis in male non obese diabetic (NOD) mice (3) and thyroiditis in biobreeding (BB) rats (4). In several other situations, chronic, long lasting inflammation can eventually lead to tissue damage. Such is the case with female NOD mice, in whom insulitis begins as early as four weeks of age, but tissue damage and islet destruction is not seen until the age of three months. In rat insulin promoter-TNF-α (RIP-TNF-α), RIP-lymphotoxin (RIP-LT), or RIP-IL-2 mice, inflammation is apparent in very young mice (before three weeks of age) but tissue damage occurs after costimulatory signals or upon antigenic stimulation (5–8).

The development and organization of chronic inflammation is just beginning to be understood. Though several cytokines and chemokines have been implicated, a clear understanding of the role of the individual factors and their mechanism of action in the inflammatory process awaits elucidation. Although the expression “tertiary lymphoid tissue” has been used to describe any tissue that can be infiltrated (9), the process of chronic inflammation had been considered to be completely different from that of lymphoid organ development.

Lymphotoxin (LT, also known as LT-α or TNF-β), a product of CD4Th1, CD8, and early B cells, has been implicated in the inflammatory process though it has many other activities as well (reviewed in 10). A major role in inflammation is implied from its ability to activate endothelial cells in vitro for the expression of the adhesion molecules ICAM-1 and VCAM-1 (11, 12). Studies with T cell clones indicate its importance (with TNF-α) in the transfer of experimental allergic encephalomyelitis (EAE) (13, 14). The

1 Abbreviations used in this paper: BB, biobreeding; EAE, experimental allergic encephalomyelitis; HEV, high endothelial venules; LT, lymphotoxin; NOD, non obese diabetic; PFC, plaque-forming cell; PLP, periodate/lysine/paraformaldehyde; RIP, rat insulin promoter.
ability of LT and TNF-α to influence EAE is due in large part to the upregulation of VCAM-1 in the CNS (15) and the extent of inflammation is positively correlated with expression of that adhesion molecule. Inhibition of LT and TNF-α in this situation prevents the upregulation of VCAM-1 and the subsequent recruitment of additional T cells, B cells, and macrophages (15).

LT induces inflammation at sites of targeted expression in transgenic animals. We have previously described mice transgenic for LT under the control of the rat insulin promoter (RIP-LT mice). This somewhat leaky promoter is expressed in the pancreatic islets of Langerhans, proximal convoluted tubules in the kidney (16, 17), and skin. The expression of LT in these circumstances results in an infiltrate consisting of T cells, B cells, and macrophages at the sites of cytokine expression and results in a ruffled hair phenotype (18). A similar inflammatory process is seen in islets of RIP-TNF-α mice (18, 19). Tissue damage does not occur spontaneously in either RIP-LT or RIP-TNF-α mice, but can be induced by coexpression in the islets of the B7-1 costimulatory molecule which activates infiltrating T cells resulting in beta cell destruction and diabetes (5, 7). The mechanism of the LT-induced inflammatory process has not been elucidated, nor have the relative contributions of the LT transgene and products of the cells in the infiltrates.

LT, in addition to its role in inflammation, plays a crucial and unique role in lymphoid development and tissue organization. This is apparent from our analysis of mice made selectively deficient in LT expression through the process of homologous recombination (20). Such LT knockout mice are devoid of all peripheral and mesenteric lymph nodes and Peyer's patches. Splenic organization is disrupted with the loss of usual compartmentalization into T and B cell areas. Since LT-deficient mice produce normal levels of TNF-α, it is apparent that this function in lymphoid organogenesis is peculiar to LT. The mechanism of LT's effect in lymphoid organogenesis has not been investigated.

Here we report the results of studies in which we evaluated RIP-LT mice to test the hypothesis that chronic inflammation represents lymphoid neo-organogenesis and that LT plays a crucial and identical role in both. We found that the mononuclear accumulations in pancreata and kidneys are similar if not identical to organized lymphoid tissue with regard to cellular composition, compartmentalization, a specialized vascular system with expression of markers characteristic of vessels in lymph nodes and ultrastructural appearance of high endothelial venules, and reactivity to an exogenous antigen. We show that even RIP-LT mice that lack the recombination activating gene 2 protein (RIP-LT/RAG-2 -/- mice) and thus any additional cytokines derived from mature T or B cells express endothelial adhesion markers, VCAM-1, ICAM-1, and MAdCAM-1, that are likely to initiate mononuclear accumulation. Therefore, we propose that LT-induced chronic inflammation is actually lymphoid neogenesis and that the cytokine recapitulates in the adult its activities in embryogenesis, in part through its ability to induce changes in the vasculature. This provides a unifying model for lymphoid development and inflammation and has implications for determinant spreading and clinical exacerbation in autoimmune disease.

Materials and Methods

Mice. The generation of RIP-LT transgenic mice has been described (17). Animals were backcrossed to C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) or to mice lacking the RAG-2 gene (21). A breeding nucleus of the latter was kindly provided by Dr. Frederick Alt and maintained under specific pathogen-free conditions. These mice fail to produce mature B or T lymphocytes. The presence of the RIP-LT transgene and of the RAG-2 deletion was determined by Southern blot hybridization as described (17, 21). FACS analysis for the presence of CD3+ cells in peripheral blood was performed to ascertain the absence of mature lymphocytes in the RAG-2 -/- animals. To achieve maximal transcription from the insulin promoter, some animals were kept on a high-fat diet and given 10% sucrose in drinking water.

Primary Antibodies for FACS and Immunohistochemistry. The following primary antibodies were used for immunohistochemistry or for flow cytometry: YN 1/1.7 (specific for ICAM-1 [22]), MK-2 (specific for VCAM-1 [23]), RA3-6B2 (specific for the B-cell restricted determinant of B220; Caltag Laboratories, South San Francisco, CA); F4/80 (specific for macrophages); anti-CD8α-Phycoerythrin (PE; GIBCO BRL, Gaithersburg, MD); anti-CD4-PE (GIBCO BRL); FITC-labeled anti-mouse immunoglobulin (Sigma); N418 (specific for the p150/90 αβ integrin expressed on dendritic cells (24); a kind gift of Dr. Ralph Steinman); FDC-M1 (specific for mouse follicular dendritic cells (25); a kind gift of Dr. M.H. Kosco-Vilbois); PE-anti-mouse CD3-ε (Pharmingen, San Diego, CA); MECA-367 (specific for MAdCAM-1 [26]); MECA-79 (specific for PNAd [27]), and MJ 7/18 (specific for endoglin [28]) were generously provided by Dr. Eugene Butcher.

Flow Cytometry. Cells were isolated from lymph nodes and kidneys by gentle pressure with microscope slides and washed three times in Hanks' medium. Lymphocytes were purified from the kidney preparations by centrifugation over Histopaque-1090 (Ficoll/Hypaque; Sigma). Cells were washed once with staining buffer (5% fetal calf serum, 10 mM sodium azide in PBS), and incubated with the appropriate biotinylated primary antibodies in 3% mouse globulin (Sigma); N418 (specific for the p150/90 αβ integrin expressed on dendritic cells (24); a kind gift of Dr. Ralph Steinman); FDC-M1 (specific for mouse follicular dendritic cells (25); a kind gift of Dr. M.H. Kosco-Vilbois); PE-anti-mouse CD3-ε (Pharmingen, San Diego, CA); MECA-367 (specific for MAdCAM-1 [26]); MECA-79 (specific for PNAd [27]), and MJ 7/18 (specific for endoglin [28]) were generously provided by Dr. Eugene Butcher.

Immunohistochemistry. Tissues were fixed in periodate/lysine/paraformaldehyde (PLP) (29) fixative overnight, processed through three consecutive sucrose solutions (10, 20, and 30%), and snap frozen in Tissue Tek compound (Miles, Elkhart, IN). Tissue sections were cut with a Cryocut 1800 cryostat (Reichert Young, Deerfield, IL) at 7 μm and stored at −20°C. Sections were air dried and rehydrated for 10 min in wash buffer (0.1 M phosphate buffer, pH 7.3, with 0.01% Triton X). To prevent background
staining from endogenous peroxidase and nonspecific binding of antibodies, sections were treated with 0.3% H₂O₂ in methanol and with 10% normal goat serum (Pierce Chem. Co., Rockford, IL). Incubation with the primary antibody was performed for 1–3 h. After three rinses with wash buffer, an appropriate biotinylated species-specific secondary antibody ( GibCO BRL, Gaithersburg, MD) was applied. Then, after incubation with horseradish peroxidase-streptavidin (Vector Laboratories, Burlingame, CA), DAB (Sigma) was used for the color reaction. Slides were counterstained with methyl green (Sigma) and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

**Fluorescence Microscopy.** Tissues were fixed in 2% paraformaldehyde/5% sucrose for 30 min, infused with 20% sucrose for 1.5 h, and snap frozen in Tissue-Tek compound. Tissue sections were cut with a Cryocut 1800 cryostat at 5 μM and stored at −20°C. Sections were air dried, fixed in acetone for 10 s, and rehydrated in wash buffer (PBS, pH 7.4) for 10 min. For identification of T and B lymphocytes sections were incubated for 1 h with directly conjugated monoclonal antibodies 145-2C11-FITC (anti-CD3) and RA3-6B2-PE (anti-B220) (PharMingen, San Diego, CA) in staining buffer (PBS, 2.5% Blotto (Pierce), 10% rabbit serum (Sigma)). Plasma cells were stained as above with FITC-labeled anti-mouse immunoglobulin. Stained sections were rinsed three times in wash buffer, coverslipped with Gel/Mount (Biomeda Corp., Foster City, CA) and observed by UV-light microscopy using a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY). Kodak Ektachrome P1600 film was used.

**Hematoxylin-eosin Stain.** Tissues were fixed in neutral buffered zinc-formalin, embedded in paraffin, sectioned at 5 μM, and stained with hematoxylin and eosin following standard techniques.

**Electron Microscopy.** Animals were euthanized by inhalation of Metofane (Methoxyflurane; Pitman-Moore, Inc., Mundelein, IL) and perfused with 0.1 M sodium cacodylate. After postfixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate for an additional one hour, and washed three times with 0.1 M sodium cacodylate. After postfixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate. After post fixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate. After post fixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate. After post fixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate. After post fixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate. After post fixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate. After post fixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate. After post fixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate. After post fixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate. After post fixation in 1.33% osmium tetroxide for 1 h, tissues were washed three times with 0.1 M sodium cacodylate.
Figure 1. RIP-LT transgenic kidneys resemble lymphoid organs. (A) Macrophotically, the kidney of the non-transgenic animal is pale, with many surface protrusions (left). The kidney of the non-transgenic mouse is red, with no protrusions (right). (B) H- and E-stained paraffin embedded tissue sections show that the protrusions on the surface of the RIP-LT transgenic kidney (left) are caused by massive cellular accumulations. No infiltrate is visible in a non-transgenic kidney (right). (C) Higher magnification of a RIP-LT transgenic kidney (×400). Mononuclear infiltrating cells are clearly discernible, with the appearance of primary follicles and germinal centers.

dendritic cells, were also present in the infiltrate (Fig. 2 D). As follicular dendritic cells are thought to play an important role in the maturation of B cells in lymph nodes and spleen, and interdigitating dendritic cells present antigen to T cells, the presence of these two cell types in the infiltrate of RIP-LT animals is particularly notable. In addition, fully differentiated B cells (plasma cells) were detected in the infiltrates in kidney (Fig. 2 E) and in pancreas (not shown) of SRBC-immunized RIP-LT mice. This result is suggestive of in loco maturation of the B cells.

To determine whether the infiltrating cells were present in the same proportions as in a conventional lymphoid organ, flow cytometric analysis was performed on cells isolated from RIP-LT infiltrates. We have previously compared pancreatic infiltrates of RIP-TNF-α and NOD mice by FACS analysis (18). Such studies could be criticized for the possibility of contamination by cells from peripancreatic lymph nodes. Furthermore, the number of cells obtained in this way, particularly from RIP-LT pancreata is quite small. Therefore, as we extended our study here we concentrated our analysis on lymphoid cells obtained from RIP-LT kidneys and compared them with the composition of a pool of mesenteric and peripheral lymph nodes. Mononuclear cells were stained with antibodies specific for T cells, B cells, and macrophages. The cellular composition of the kidney infiltrate was very similar to that of lymph nodes of the same animal (Fig. 3). The percentage of B cells in lymph nodes and kidneys was virtually identical. There was a somewhat lower proportion of T cells present in kidney infiltrates, while there were slightly more F4/80-positive macrophages in RIP-LT kidneys than in lymph nodes, though they were still a small percentage of the total. As expected from the higher number of CD3+ cells in lymph nodes, the absolute numbers of CD4+ and CD8+ cells was higher in lymph nodes than in kidneys. However, the ratio of CD4+ to CD8+ cells in the two organs was virtually identical. These data show that all cell types characteristic of a lymph node can be found in a chronic infiltrate caused by LT, and that the proportion of the cells in the infiltrate is very similar to that in lymph nodes.

RIP-LT Induced Inflammation Includes Vessels With Morphologic and Antigenic Characteristics of Those in Lymph Nodes. To determine whether the presence of LT leads to the development of vessels characteristic of lymph nodes, we evaluated tissues from RIP-LT mice and transgene-negative littermates morphologically in the electron microscope and histochemically for the expression of markers characteristic of lymph node vessels. In these studies we concentrated on the pancreas as this allowed a comparison with previous studies of vessels in the pancreatic infiltrates in NOD mice (18, 31, 32). Furthermore, the location of LT-expressing β cells and the infiltrates are more easily defined in the pancreas than in the kidney. High endothelial venules (HEV) are specialized vessels, usually present in lymph nodes, which are the sites of migration of lymphocytes from the bloodstream into the lymphoid organ. Lymph node HEV have a characteristic ultrastructural morphology, with an increase in vessel wall thickness, protrusions into the lumen of the vessels, and enlarged, plump nuclei, and they express peculiar adhesion molecules called addressins.

Endothelial cells in vessels in the vicinity of endocrine cells in the pancreas of transgene-negative controls had the
morphology typical of a non-inflamed pancreas. The vessel wall was smooth and lacked protrusions and exhibited elongated nuclei (Fig. 4 A). Blood cells are not obvious in the vessels of these extensively perfused mice. Vessels in pancreata of RIP-LT mice showed the typical morphology of HEV (12, 33) with an irregular and increased thickness of the vessel wall, protrusions into the vessel lumen, and enlarged and plump nuclei (Fig. 4 B). These findings demonstrate that LT expression leads to the development of high endothelial venules. These results therefore indicate that the expression of LT at a local site results in the differentiation of vessels into HEV. As HEV are the sites of migration of lymphocytes from the bloodstream into lymphoid organs, this suggests that changes in endothelial cells could contribute to the development of the infiltrates in RIP-LT mice.

A complex system of vascular addressins is responsible for lymphocyte extravasation into lymphoid organs. We investigated whether the adhesion molecules that are expressed on vessels in lymphoid tissues are also expressed in the regions of LT-induced chronic inflammation. The adhesion molecules PNAd (peripheral node addressin) and MAdCAM-1 (mucosal addressin–cell adhesion molecule 1) are involved in the recirculation of lymphocytes into lymphoid
Figure 3. Flow cytometric analysis for Ig, CD3, F4/80, CD4, and CD8. Cells were obtained from a pool of mesenteric and peripheral lymph nodes and from the kidney of a RIP-LT transgenic animal and stained with the indicated antibodies as described in Experimental Procedures. The intensity of staining and relative percentages of B cells, T cells, and macrophages were very similar in lymph node and transgenic kidney. The ratio of CD4+ cells to CD8+ cells in the kidney is the same as in a lymph node. The somewhat higher percentage of F4/80+ cells in the kidney infiltrates could be due to staining of mesangial cells. Solid line indicates staining with an irrelevant control antibody; dotted line indicates staining with anti-Ig, anti-CD3, anti-F4/80, anti-CD4, or anti-CD8.

Lymph Node Kidney

|       | Lymph Node | Kidney |
|-------|------------|--------|
| B cells | 38.3%      | 44.1%  |
| T cells | 47.6%      | 31.3%  |
| CD4/CD8 ratio | 1.3       | 1.2    |
| Macrophages | 0.73%     | 2.79%  |

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...and mucosal tissues (26–28). In the adult, PNAd is expressed predominantly on endothelial cells in peripheral lymph nodes; MAdCAM-1 is found mainly in mesenteric lymph nodes and Peyer’s patches. To determine if these adhesion molecules were expressed in the pancreata of RIP-LT mice, immunohistochemical studies were performed. Though MAdCAM-1 was expressed constitutively at low levels in the exocrine pancreas of RIP-LT-negative mice, it was clearly upregulated on endothelial cells of vessels within the infiltrate in RIP-LT mice. This upregulation was restricted to vessels within the peri-insular infiltrate, and was not seen on vessels within the RIP-LT islets (Fig. 5, A and B). PNAd was not detected on any vessels in the pancreata of non-transgenic mice (Fig. 5 C), but was present on vessels located within the chronic infiltrate of the peri-insular lesions of RIP-LT animals. As was true with MAdCAM-1, PNAd expression was restricted to vessels within the infiltrate. Endothelial cells located within the islets of RIP-LT animals did not express PNAd (Fig. 5 D). These studies also suggest that LT expression resulted in angiogenesis. For example, in Fig. 5 B, a larger vessel, that does not express MAdCAM-1, is present in close proximity to an islet. A smaller vessel, highly positive for MAdCAM-1, is growing out of this larger vessel and branching out into the location of the infiltrate. Immunohistochemical analysis with an antibody specific for endoglin, a marker expressed by most endothelial cells (28), also indicated angiogenesis in a peri-insular and intra-insular location in RIP-LT mice (data not shown).

The Cells of the Infiltrate Can Respond to an Exogenous Antigen. While the presence of LT was sufficient for the development of the morphologic appearance and cellular composition of a lymphoid organ, the central property of organized lymphoid tissue is its ability to generate an immune response. We first studied whether cells isolated from RIP-LT kidneys and pancreata could respond to T and B cell mitogens. A significant proliferative response was induced by the T cell mitogen Con A and the B cell mitogen LPS (data not shown). In order to investigate the ability of the LT-induced infiltrate to generate an immune response to an exogenous antigen, RIP-LT mice were immunized with SRBC and lymphocytes were obtained from lymph nodes, spleens, and kidneys. (It was not possible to obtain sufficient cells from pancreata for these studies.) Direct plaque-forming cell (PFC) assays performed to evaluate the presence of cells producing IgM to SRBC revealed that in lymphocytes obtained from kidneys from SRBC immunized RIP-LT mice, the number of PFC was comparable to that in lymph nodes (Table I). To determine whether IgG production and isotype class switching occurred in the lymphoid accumulations in RIP-LT kidneys, indirect PFC assays were performed with cells from SRBC boosted RIP-LT mice. IgG-SRBC PFC were present in the kidneys of RIP-LT mice (Table I). These experiments show that the newly formed “lymphoid organs” in the kidneys of RIP-LT animals are able to generate an immune response, as shown by their ability to produce SRBC-specific IgM-producing cells and to provide T cell help to perform immunoglobulin class switching.

LT, Even in the Absence of Mature Lymphocytes, Induces Several Characteristics of a Lymphoid Organ. As indicated above, the expression of LT in tissues of immunocompetent animals...
Figure 4. RIP-LT induced inflammation includes vessels with the morphologic characteristics of HEV. (A) In the electron microscope, vessels from a non-transgenic animal show even, thin vessel walls. No protrusions are seen. (B) Vessels from a RIP-LT transgenic animal show protrusions into the lumen and thickening of the endothelial cell body. (© endocrine cells; *, pancreatic duct; arrows indicate endothelial cells.) (6,000 X).

resulted in infiltrates consisting of all cell types normally seen in a lymphoid organ. The development of these inflammatory lesions could be the direct effect of LT, or could be partly mediated by factors derived from the infiltrating cells. To gain insight into the mechanism of LT's induction of lymphoid cell accumulation in the absence of lymphocytes, and to determine whether factors derived from mature lymphocytes were necessary for the induction of adhesion molecules to occur, we evaluated RIP-LT RAG-2 -/- mice. These mice, shown to be homozygous for the RAG-2 knockout mutation by Southern blot analysis, showed a complete absence of CD3+ T cells in FACS analysis of peripheral blood. Immunohistochemical analyses of markers expressed on CD4+ and CD8+ T cells, B cells, macrophages, dendritic cells and follicular dendritic cells were carried out on pancreatic tissue sections of RIP-LT RAG-2 -/- mice. Only a few inflammatory cells were present in the kidneys and pancreas of RIP-LT RAG-2 -/- mice. These were mainly dendritic cells, follicular dendritic cells, macrophages, and a few immature lymphocytes (data not shown). Therefore, the elicitation of even a minimal infiltrate was not dependent on factors derived from mature lymphocytes. Because these studies establish that hardly any inflammatory cells are present at the sites of LT expression in RIP-LT RAG-2 -/- mice, these animals are appropriate to analyze the effects of LT in the absence of a major inflammatory lesion.

RIP-LT RAG-2 -/- mice were investigated to determine whether LT directly induces expression of adhesion molecules in vivo. Previous studies have demonstrated its ability to induce VCAM- and ICAM-1 on endothelial cells in vitro (11, 12), and its close relative, TNF-α has been shown to also induce MAdCAM-1 in vitro (34). We have previously reported that the adhesion molecules ICAM-1 and VCAM-1 are upregulated in RIP-LT mice on vessels within islets and in the peri-insular space. However, ICAM-1 is also expressed on some of the infiltrating cells (18). The high levels of ICAM-1 and VCAM-1 on vessels in the islets even in areas lacking infiltrates suggests that the transgene-derived LT, rather than factors derived solely from infiltrating cells, induces these molecules. To critically address the role of LT in ICAM-1 and VCAM-1 upregulation, adhesion molecules were evaluated in pancreata of RIP-LT RAG-2 -/- mice and their non-transgenic littermates. The low constitutive expression of ICAM-1 in non-transgenic RAG-2 -/- mice was comparable to that observed in non-transgenic immunocompetent animals. In RIP-LT RAG-2 -/- animals, there was clear upregulation of the adhesion molecule on the endothelial cells of vessels throughout the islets, in the peri-insular space, and in the exocrine tissue in close vicinity to the islets (Fig. 6 A). The increased ICAM-1 expression was comparable to that in immunocompetent RIP-LT mice. Almost no expression of VCAM-1 was observed in RAG-2 -/- non-transgenic pancreatic tissues. This confirmed our previous report in non-transgenic immunocompetent mice (18). Upregulation of VCAM-1 was observed in RIP-LT-positive RAG-2 -/- pancreata (Fig. 6 B). The distribution of VCAM-1 was the same as that of ICAM-1. In order to quantify the upregulation of ICAM-1 and VCAM-1, coded tissue sections stained for ICAM-1 and VCAM-1 were scored as showing normal or increased expression of the adhesion molecules. Increased expression of ICAM-1 was seen in 100% of the islets and VCAM-1 in 98% of the islets in RIP-LT-positive mice. The difference
between adhesion molecule expression in islets of RIP-LT-negative and -positive mice is statistically significant ($P < 0.005$ by ANOVA). These findings demonstrate that additional factors derived from mature lymphocytes are not necessary for the upregulation of ICAM-1 and VCAM-1 by LT and suggest that LT can induce their expression.

Vessels in RIP-LT RAG-1-/- pancreata were studied for markers more typical of lymph nodes. They had the typical morphology of HEV (not shown) indicating that the presence of LT is sufficient to cause the morphologic differentiation of normal vessels into HEV. As we have shown previously that all $\beta$ cells in RIP-LT animals express the transgene (17), the expression of MAdCAM-1 and PNAd, which was restricted to the sites of inflammation, rather than throughout the islets (Fig. 5), suggested either a role for the infiltrating mononuclear cells in the upregulation of these molecules, or a difference in the susceptibility of vessels in different anatomical locations to the effects of LT. In RIP-LT RAG-1-/- mice, there was additional expression of MAdCAM-1 on the vessels in the peri-vascular space (Fig. 6 C). Therefore, LT in the absence of mature lymphocytes also induced increased expression of MAdCAM-1. The highly restricted expression of PNAd in the location of the infiltrate in immunocompetent RIP-LT animals (Fig. 5) suggests the possibility that the infiltrating cells had a causative role in the upregulation of this adhesion molecule. The fact that PNAd was not detected in RIP-LT pancreata from animals that lacked mature lymphocytes (Fig. 6 D) indicates that, in contrast to the observations with VCAM-1, ICAM-1, and MAdCAM-1, addi-

### Table 1. Plaque Forming Cells in Kidneys of RIP-LT Mice Immunized with SRBC

| Organ          | Direct (IgM) | Indirect (IgG) |
|----------------|--------------|----------------|
| Kidney         | 421          | 2,357          |
| LN (cervical)  | 470          | 5,370          |
| LN (mesenteric)| 2,210        | 6,109          |
| Spleen         | 1,540        | 15,160         |

Mice were immunized as indicated in Materials and Methods with $2 \times 10^6$ SRBC i.v., boosted 10 d later with the same dose i.p., and killed 6 d later. Data shown are representative of four experiments with similar results. No lymphocytes were present in kidneys of RIP-LT negative animals, and no PFC were obtained from kidneys of non-immunized RIP-LT mice.

Figure 5. RIP-LT induced inflammation includes vessels with biochemical characteristics of lymphoid tissues. (A) Very low constitutive expression of MAdCAM-1 on vessels in the exocrine pancreas of a transgene negative animal. (B) Increased expression of MAdCAM-1 on vessels in the infiltrate of a RIP-LT transgenic animal. Note that the vessel expressing MAdCAM-1 appears to be growing out of a larger vessel, suggesting the possibility of angiogenesis. (C) No PNAd expression on vessels in the pancreas of a non-transgenic littermate control. (D) PNAd expression in vessels in the chronic infiltrate of a RIP-LT animal. (125X).
Discussion

Our data support a unifying model for lymphoid organ development and chronic inflammation. We show that the chronic inflammation caused by local expression of LT has all the characteristics of functional lymphoid tissue. All cell types normally found in lymphoid organs, including T cells, B cells, plasma cells, follicular dendritic cells, and other antigen-presenting cells, were present in the RIP-LT-induced infiltrates. The proportions of these cells were very similar to those observed in a lymph node. In addition to T and B cell compartments, areas with the morphological appearance of primary and secondary follicles were present. The endothelial cells of the vessels in the infiltrates had the typical morphology of high endothelial venules and expressed ICAM-1 and VCAM-1, molecules usually associated with chronic inflammation, and PNAd and MAdCAM-1, molecules normally associated with vessels in peripheral or mesenteric lymph nodes. In addition to the morphologic characteristics of organized lymphoid tissue, the cells of the chronic infiltrate showed the functional attributes of a lymphoid organ in their ability to generate a T cell-dependent antibody response and to induce class switching. Lymphocytes in the newly organized lymphoid accumulations in the pancreata and kidneys of RIP-LT mice are most likely derived from the recirculating pool of mononuclear cells, just as they are in normal lymph nodes. The organization into germinal centers and the presence of plasma cells points to a local activation of these cells. Studies with RIP-LT transgenic mice that lack secondary lymphoid organs are under way to further investigate this question.

The LT-induced upregulation of the adhesion molecules ICAM-1, VCAM-1, and MAdCAM-1 and the morphologic appearance of HEV did not depend upon factors derived from mature lymphocytes. On the other hand, mature lymphocytes, in addition to the transgene product, were necessary for the expression of PNAd. These findings suggest that many of the effects of LT in lymphoid neogenesis are direct effects of the cytokine, as suggested by in vitro studies (11, 34) or secondary to its expression, and not caused by factors derived from infiltrating lymphoid cells. Other effects most likely require chemokines and other cytokines derived from local and infiltrating cells and are secondary or even independent of LT.
Further studies are needed to explore the interactions of other cytokines and chemokines with LT in lymphoid neo-organogenesis. There are several transgenic models in which local expression of cytokines leads to the development of inflammatory lesions that in many respects are similar to those described here. Mice transgenic for TNF-α, IL-2, or IL-10 under the control of the insulin promoter show pancreatic inflammation, but do not develop spontaneous diabetes (18, 19, 35, 36) and mice expressing IFN-α or IFN-γ under the same promoter develop both inflammation and diabetes (37–39). Mice transgenic for a modified TNF-α gene develop arthritis (40). It is not known whether the infiltrates in these systems also exhibit all the lymphoid organ characteristics that RIP-LT mice do and whether and how LT participates. It is possible that LT and other cytokines induce converging pathways that contribute to these inflammatory events. It is quite possible that LT plays a critical role in these systems, because of all the cytokine or cytokine receptor knockout mice (including those lacking the p55 and/or p75 TNF receptors), only LT-deficient mice lack lymph nodes. It is therefore very probable that LT's role in the induction of lymphoid organs is unique. Answers to these questions may be provided in the analysis of e.g., RIP-IL-2 LTα-/- mice being generated now in our laboratory.

Our work provides a functional implication and mechanism for the phenomena that have given rise to the term "tertiary lymphoid tissue." This designation has been suggested for all tissues because they can be induced to recruit unique subsets of lymphocytes in the setting of inflammation (9). Consistent with this designation have been several previous reports that chronic inflammatory lesions resemble lymphoid tissues. Clearly delineated T and B cell areas can be seen in inflamed synovium of patients suffering from rheumatoid arthritis (41) and this tissue produces the same immunoglobulin isotypes as does a lymphoid organ (42). Vessels with the characteristics of HEV have been described in the lesions of experimental allergic encephalomyelitis (43–46). Thyroiditis in BB rats is so similar to a secondary lymphoid organ that it has been suggested that this inflammation in which very little tissue destruction occurs should be called "thyroid associated lymphoid tissue" (4). Vessels in the insulitis of prediabetic NOD mice express MAdCAM-1 and PNAd (31, 32). The characteristics of lymphoid neogenesis noted above in several different types of chronic inflammatory lesions indicate that this process occurs throughout life in adults and is not an isolated phenomenon peculiar to the transgenic system described here. The functional implication of the designation "tertiary lymphoid tissue," namely the possibility that antigen can be presented at a local inflammatory site and contribute to determinant spreading (47), is apparent from our work and provides an appreciation that the mechanism of inflammation may be very similar to the process of fetal lymphoid organogenesis.

Our work provides a framework to consider common mechanisms for lymphoid organogenesis and inflammation. The first cells observed in lymphoid organ development are lymphocytes that migrate from veins to the sites of the future lymph nodes (48). Though most studies evaluating LT regulation have concentrated on CD4+Th1, CD8+, or pre-B cells (49, 50), and virus infected cells (51), LT can also be made by a variety of other cell types, including NK cells and astrocytes (10). Therefore, there are several possible sources of LT that could contribute to lymph node formation in embryonic development. LT in development (whatever its source) and inflammation (from T cells) could cause the infiltration of mononuclear cells via upregulation of adhesion molecules and other changes in endothelial cells. The data presented here clearly show that LT alone is sufficient for the expression of the adhesion molecules ICAM-1, VCAM-1, and MAdCAM-1 and the morphologic changes characteristic of the high endothelial venules found in lymphoid organs, and eventually leads to cellular and functional attributes of a lymphoid organ.

The findings reported here provide insight into mechanisms that lead to lymph node development and of those forms of chronic inflammation that lead to the formation of lymphoid tissues in inappropriate locations. They imply that one cytokine, LT, not only plays a crucial role in these processes, but that it is sufficient for many of their associated characteristics. Our work revealing LT as an initiating factor in chronic inflammation provides a clearer understanding of these events. An even better understanding will come from our ongoing studies of RIP-LT mice that lack any endogenous LT (LT-α-/- mice) and from the elucidation of the role of receptors, signaling pathways, and other LT-induced proteins. Such studies could lead to highly specific therapies for a variety of diseases. For example, specific LT-antagonists could be useful for the prevention of determinant spreading in autoimmune diseases. Engineered release of LT at sites of malignant lesions could lead to inflammation and provide a local milieu to induce an immune response against a tumor. Elucidation of the mechanisms of lymphoid organogenesis could also be important for the therapy of other diseases as well. Patients who receive whole-body irradiation often develop an atrophy of their lymphoid tissues; presently, few therapeutic modalities exist to influence the regeneration of these organs. It is possible that LT could be useful here. The lesions of pulmonary lymphoid hyperplasia seen in pediatric AIDS patients have characteristics of a lymphoid organ (52, 53) hinting at a role of LT and therapeutic implications of the findings reported here for some infectious diseases.

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