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Expression of the lymphotoxin β receptor on follicular stromal cells in human lymphoid tissues

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Abstract

The lymphotoxin β receptor (LTβR), and its ligand, LTα1β2, have been proposed to play a key role in the development and organization of lymphoid tissues. The LTβR is expressed on a variety of human primary and transformed cells, but strikingly absent on T or B lymphocytes and primary monocytes or peripheral dendritic cells, although LTβR is detected on some myeloid leukemic lines. In the developing thymus LTβR is prominent along the trabeculae and into the medulla upto corticomedullary junction. In the spleen, LTβR is prominently expressed by cells in the red pulp and along the borders of red and white pulp which colocalizes with reticular stromal cells. The LTβR is expressed on a human follicular dendritic cell line, FDC-1, and signals expression of CD54 when ligated with the LTα1β2 complex. These results support the concept that directional interactions between LTα1β2 bearing lymphocytes and LTβR bearing stromal cells are involved in the organization of lymphoid tissue.

Keywords: thymus, spleen, reticular dendritic cells, follicular dendritic cells, lymphoid organization, ICAM-1

Abbreviations: C, cortex; DC, dendritic cells; FDC, follicular dendritic cell; H, Hassall's corpuscle; LTα, lymphotoxin α; LTβ, lymphotoxin β; LTβR, lymphotoxin β receptor; M, medulla; R, red pulp; T, trabeculae; TNFR, tumor necrosis factor receptor; V, blood vessel; W, white pulp

Introduction

The lymphotoxin β receptor (LTβR) is a member of the TNF receptor family that has been proposed to play a role in the development and organization of lymphoid tissues (Beutler and vanHuffel, 1994; Ware *et al*, 1995). Unlike the other TNF receptors (TNFR), LTβR does not bind soluble ligand, but rather, a membrane form of lymphotoxin (LT) (Crowe *et al*, 1994) comprised of one LTα and two LTβ molecules (LTα1β2) (Androlewicz *et al*, 1992; Browning *et al*, 1993; 1995). The LTβ subunit is a 33 kDa type II transmembrane protein that serves as an anchor for LTα, which lacks a transmembrane domain (Androlewicz *et al*, 1992; Browning *et al*, 1991; 1993; Ware *et al*, 1992). No secreted form of LTα1β2 has been identified, implying that signaling through the LTβR/LTα1β2 interaction requires cell to cell contact (Browning *et al*, 1995). LTα is also secreted as a homotrimer which binds either the 55–60 kDa TNFR (TNFR60, CD 120a) or the 75–80 kDa TNFR (TNFR80, CD120b) (Beutler and vanHuffel, 1994; Ware *et al*, 1995; Vilcek and Lee, 1991). In contrast, LTα1β2 does not bind TNFR60 or TNFR80 (Crowe *et al*, 1994; Browning *et al*, 1993) and LTβR does not bind to secreted LTα homotrimer or to secreted or membrane forms of TNF (Crowe *et al*, 1994). Thus, the LTβ subunit provides the distinct LTβR specificity to the cell surface LTα1β2 heterotrimer. Although similar to TNFR60 and TNFR80 in its extracellular ligand binding domain, LTβR exhibits little sequence homology with these receptors in its cytoplasmic domain. However, functional similarities between LTβR, TNFR80 and CD40 are suggested by the findings that these receptors bind to a related set of signaling molecules, called TRAFs, which constitute a family of zinc ring finger proteins (Mosialos *et al*, 1995; Nakano *et al*, 1996). LTβR induces a slow apoptotic death in certain adenocarcinomas and tumor growth arrest *in vivo* (Browning *et al*, 1996b). Cell death induced by LTβR is dependent on TRAF3 (VanArsdale *et al*, 1997), whereas NF$\varkappa$B activation appears to be mediated by TRAF5 or TRAF2 (Nakano *et al*, 1996).

While the biological activities and signaling cascades elicited by TNF or LTα homotrimers binding to TNFR60 and TNFR80 have been extensively examined, less is known regarding the function of the LTβR/LTα1β2 interaction. The earliest hint to its function comes from findings that mice rendered deficient in LTα by targeted gene disruption display a loss of T and B cell organization in the spleen, an absence of Peyer's patches and lymph nodes and deficient germinal center formation with a conspicuous absence of follicular dendritic cells (FCD) and IgG production upon antigenic challenge (De Togni *et al*, 1994; Banks *et al*, 1995; Matsumoto *et al*, 1996a; 1996b; Fu *et al*, 1997). Some, but not all, of these phenotypic characteristics have been observed in mice with deficiencies in TNFR60 (Matsumoto *et al*, 1996b; Pfeffer *et al*,

1993; Rothe *et al*, 1993; Pasparakis *et al*, 1997), but not in TNFR80 deficient mice (Erickson *et al*, 1994), suggesting that the LTα homotrimer is not sufficient for the proper organization and function of these tissues. Thus, the binding of LTα1β2 to LTβR is implicated to play a role in lymphoid organogenesis and functional organization of lymphoid tissues. This idea is supported by recent studies demonstrating that LTβ deficient mice lack Peyer's patches, peripheral lymph nodes, splenic germinal centers and follicular dendritic cells, but retain normal cervical and mesenteric lymph nodes (Koni *et al*, 1997; Alimzhanov *et al*, 1997) and that experimental mouse systems using soluble LTβR:Fc fusion protein result in deficient splenic architecture (Ettinger *et al*, 1996) and peripheral lymph node formation (Rennert *et al*, 1996). Despite this strong evidence, little is known about the cells which express LTβR. Therefore, we examined the expression of LTβR on a variety of primary human cells and cell lines and developing human lymphoid tissues to identify the cell types involved in the control of lymphoid organization by the LTβR/LTα1β2 interaction.

Results

Comparison of LTβR expression to other TNF receptor family members

Using immunofluorescence and flow cytometry, we examined the cell surface expression of LTβR on a range of human cells of hematopoietic and non-hematopoietic origin and compared its expression to that of TNFR80, TNFR60, CD40 and Fas (CD95) (Table 1). In contrast to TNFR80, or Fas, LTβR is not expressed on resting or activated T cells or T leukemias. Both monoclonal and polyclonal goat antibodies to LTβR failed to react with T cells, excluding an epitope loss artifact. In contrast to CD40, LTβR is not expressed on an EBV transformed B cell line or a B cell lymphoma. Moderate expression of LTβR was detected on a number of leukemia cell lines, such as U937, HL60 and K562, which represent cells at various stages of myeloid differentiation. It was also found on several cell types of nonhematopoietic origin, such as the high level of expression found on ME-180 cervical carcinoma cells. This pattern of expression is similar, but not identical to that found for TNFR60.

Expression of LTβR on peripheral blood monocytes and dendritic cells (DC)

In light of our finding that several human myeloid leukemia lines express LTβR we also performed a careful analysis of LTβR expression on monocytes and DC obtained by elutriation from human peripheral blood. Figure 1A demonstrates the two populations of cells present in elutriated fractions of PBMC enriched for monocytes/DC. These fractions can be divided into CD33+, CD14− and CD33+, CD14+. We used three-color immunofluorescence to evaluate expression of CD83, HLA-DR and LTβR on these two populations immediately (day 0) or after 2 days in culture (Figure 1B). As previously described (Zhou and Tedder, 1995; O'Doherty *et al*, 1993), CD33+, CD14− DC express CD83 after 2 days in culture, while CD33+, CD14+ monocytes fail to express this marker. Both populations express HLA-DR, but the DC express high levels of HLA-DR which are markedly enhanced following 2 days of culture. In contrast, neither population expresses LTβR at either time point.

Expression of LTβR in developing human thymus

Given that T cells are the predominant source of the LTα1β2 ligand (Androlewicz *et al*, 1992; Browning *et al*, 1991; Ware *et al*, 1992) and our previous finding that mRNA for LTβR is

Table I Expression of LTβR and Related TNF Receptors[1]

			TNF receptor expression			
	Cell type	**LTβR**	**TNFR80**	**TNFR60**	**CD40**	**Fas/CD95**
$CD4^+$ T cells	Peripheral blood[2]	−	++	−	−/+	+
$CD8^+$ T cells	Peripheral blood	−	++	−	−	+
II-23	$CD4^+$ T hybridoma	−	++	++	−	++
HUT-78	$CD4^+$ T lymphoma	−	++	++	+	+++
Jurkat	$CD4^+$ T leukemia	−	++	+	−	+
Molt4	$CD4^+$ 8^+ leukemia	−	+	+	−	+
Bcell-3	B cell EBV positive	−	++	+	+++	+++
BJAB	B cell lymphoma	−	−	+	+++	++
U937	Promonocytic leukemia	++	++	+	−	+
HL60	Promyleocytic leukemia	+	+	+	−	+
K562	Myelogenous leukemia	++	++	++	−	−
WI-38	Normal lung fibroblast	++	−	+	−	+
HEK293	Embroyonic kidney	+	−	+	−	+
ME-180	Cervical carcinoma	+++	−	+	−	++

[1]Except for peripheral blood T cells, expression of TNFR was determined by single-color immunofluorescence and flow cytometry using the mAb and methods described in Materials and Methods. Fuorescence signal (peak mean channel) > 20-fold above staining with normal mouse Ig is denoted with +++; 5−20-fold, ++; 2−4-fold, +; not detected, −. On activated T cells −/+ denotes expression of CD40 depends on individual clone of human T cells and the level of CD40 on T cells is typically 10-fold less than that seen on B cell lines [25]. [2]Peripheral blood T cell subpopulations were detected by dual-color analysis with FITC-labeled anti-CD4 or anti-CD8 and biotin conjugated anti-TNFR and PE-conjugated strepavidin. The peripheral blood T cell data represents results from seven different donors with no difference noted between genders

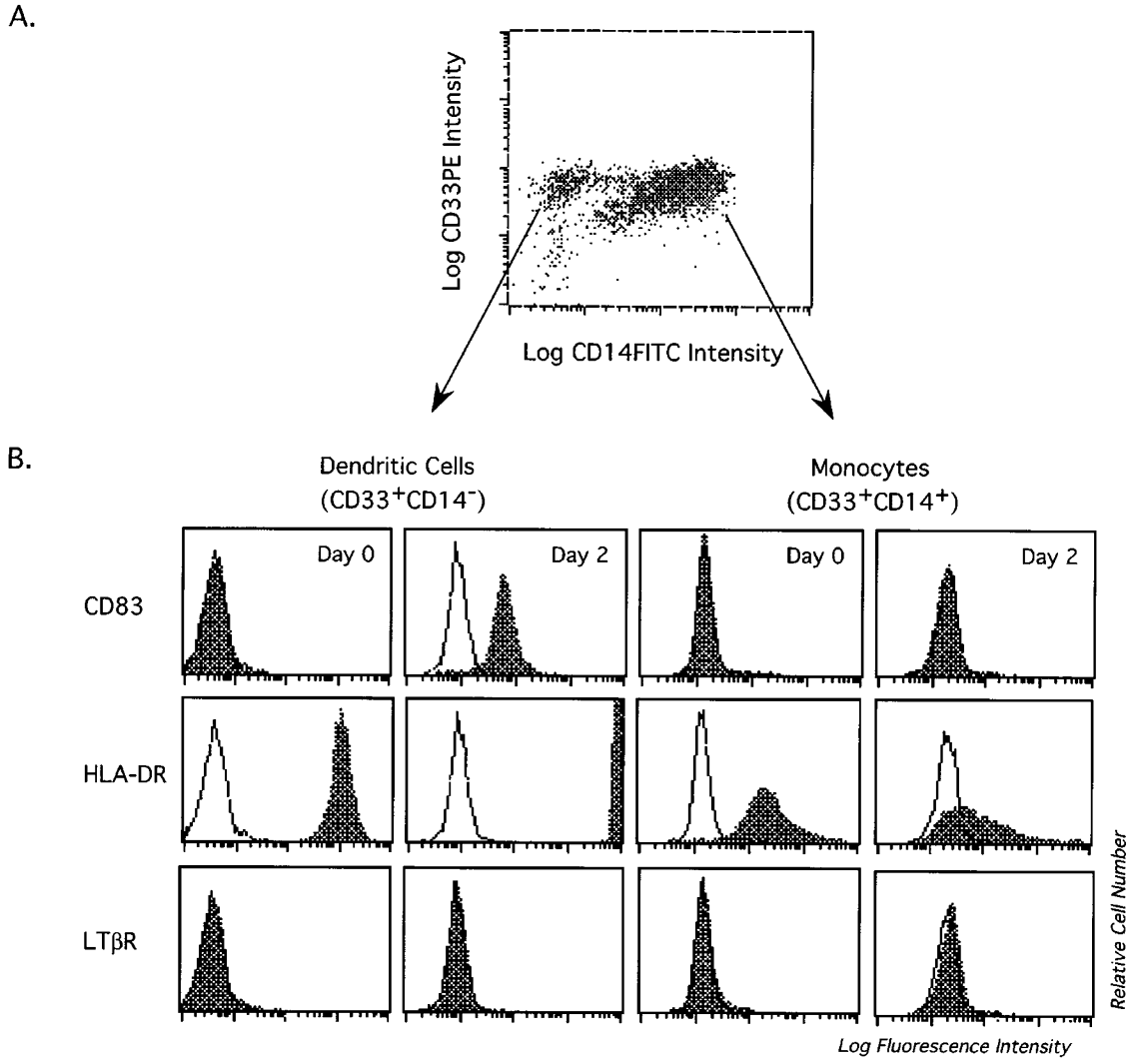

Figure 1 Analysis of LTβR expression on human peripheral blood monocytes and DC. Elutriated fractions containing peripheral blood monocytes and DC were stained with PE-conjugated anti-CD33 and FITC-conjugated anti-CD14 to differentiate between the two cell types. Monocytes (CD33+CD14+) and DC (CD33+CD14−) were individually gated and further analyzed for CD83, HLA-DR or LTβR by three-color immunofluorescence. Expression patterns were obtained immediately upon isolation and after two days in culture

expressed in a murine thymic epithelial cell line (Force *et al*, 1994), we used immunohistochemistry to examine the expression of LTβR protein in frozen sections of developing human thymus. In normal postnatal thymus (Figure 2A), LTβR expression is most prominent in the medulla (M) and along the trabeculae (T). Specific staining for LTβR is detected in the stromal rudiment of fetal thymus as early as 14 weeks gestation and distinctly localized by 16 weeks gestation. During development, the trabeculae extend through the cortex and meet with the developing medulla at the corticomedullary junction (von Gaudecker and Muller-Hermelink, 1980; Kendall, 1989). This is illustrated in Figure 2B, where LTβR expressing cells are apparent in the trabeculae, and within the medulla along the corticomedullary junction of a 20 week gestation thymus. In normal thymus, no significant changes in the level or localization of LTβR expression were observed from 20 weeks gestation to 22 months postnatal.

Colocalization of LTβR with reticular stromal cells in human thymus

To define the stromal cell types expressing LTβR we stained serial sections of postnatal human thymus with antibodies to reticular dendritic cells, LTβR or CD11c, which reacts with tissue macrophages (Lanier *et al*, 1985; Myones *et al*, 1988). The results are shown in Figure 3, where the pattern of staining for LTβR (**B**) is similar to that for reticular dendritic cells (**A**), but not that for CD11c (**C**). Most notable is the staining along the trabeculae, which are lined with reticular

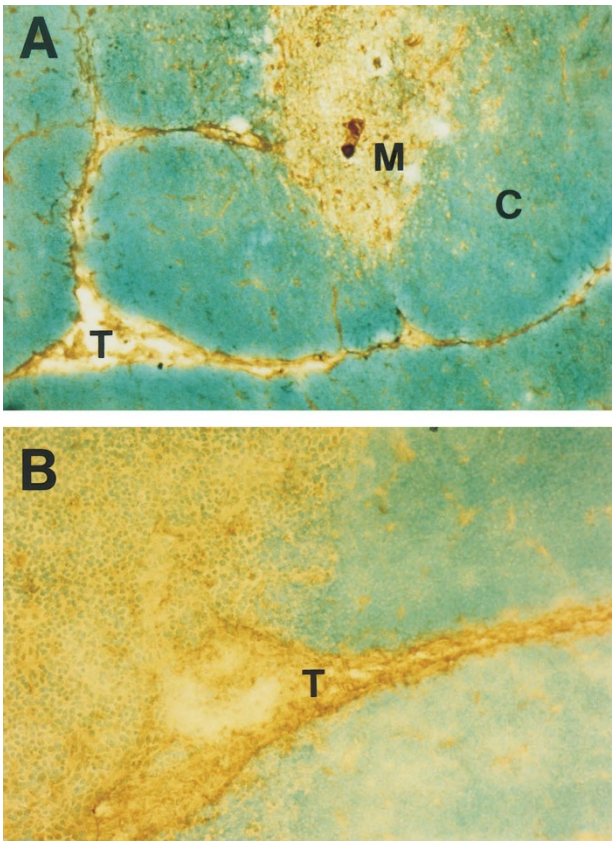

Figure 2 Expression of LTβR in developing human thymus. Frozen sections of thymus were stained for LTβR expression (brown) and counterstained with methyl green. Sections of thymus are from (**A**) a 2 month old infant (145×), (**B**) a 20 week gestation fetus (290×) Cortex (C), medulla (M) and trabeculae (T) are indicated

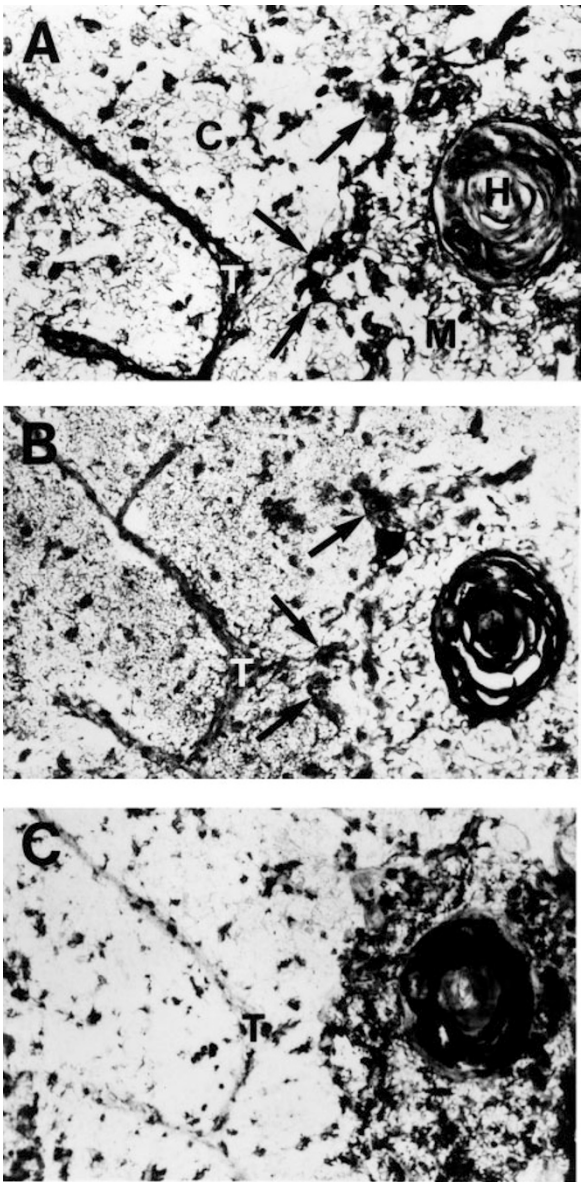

Figure 3 Colocalization of LTβR and reticular dendritic cells in human thymus. Serial sections of frozen thymus from a 7 month old child were stained for (**A**) reticular dendritic cells, (**B**) LTβR or (**C**) CD11c positive tissue macrophages. Positive staining is indicated by black. All sections are at 580×. Cortex (C), medulla (M), trabeculae (T) and Hassall's corpuscle (H) are identified. Arrows indicate positively stained cells along the corticomedullary junction

dendritic cells (von Gaudecker and Muller-Hermelink, 1980; Kendall, 1989), and the corticomedullary junction (arrows) where LTβR and the reticular dendritic cell antigens colocalize. In contrast, CD11c reacts with isolated cells along the trabeculae and numerous cells throughout the medulla. Sections of thymus stained with control goat serum or isotyped matched control mAbs showed no staining in cortex, medulla or trabeculae, and only faint, non-specific staining in Hassall's corpuscles.

Expression of LTβR in developing human spleen and colocalization with reticular dendritic cells

Given the splenic disorganization observed in LTα deficient mice we examined LTβR expression on frozen sections from ten samples of human fetal spleen ranging from 14–22 weeks gestation. Expression of LTβR was observed throughout the splenic tissue as early as 14 weeks gestation. By 20 weeks, the developing spleen shows distinct regions of red pulp (R) and lymphocyte-rich white pulp (W). LTβR is more prominently observed in the red pulp (Figure 4A) and at high power it can be seen concentrated at the R/W border in a stromal pattern of expression (Figure 4B). Sections of spleen stained with a control goat serum showed no stromal staining and only faint, non-specific staining in central arterioles (Figure 4C). We also examined serial sections of spleen for expression of reticular dendritic cells with anti-LTβR and CD11c (Figure 5). In this 17 week fetal spleen clear demarcation of the red and white pulp had not yet occurred. Specific staining for LTβR is present on stromal cells throughout the tissue (**B**) and the pattern of expression is similar to that of the reticular dendritic cell antigen (**A**), but not for CD11c positive tissue macrophages (**C**).

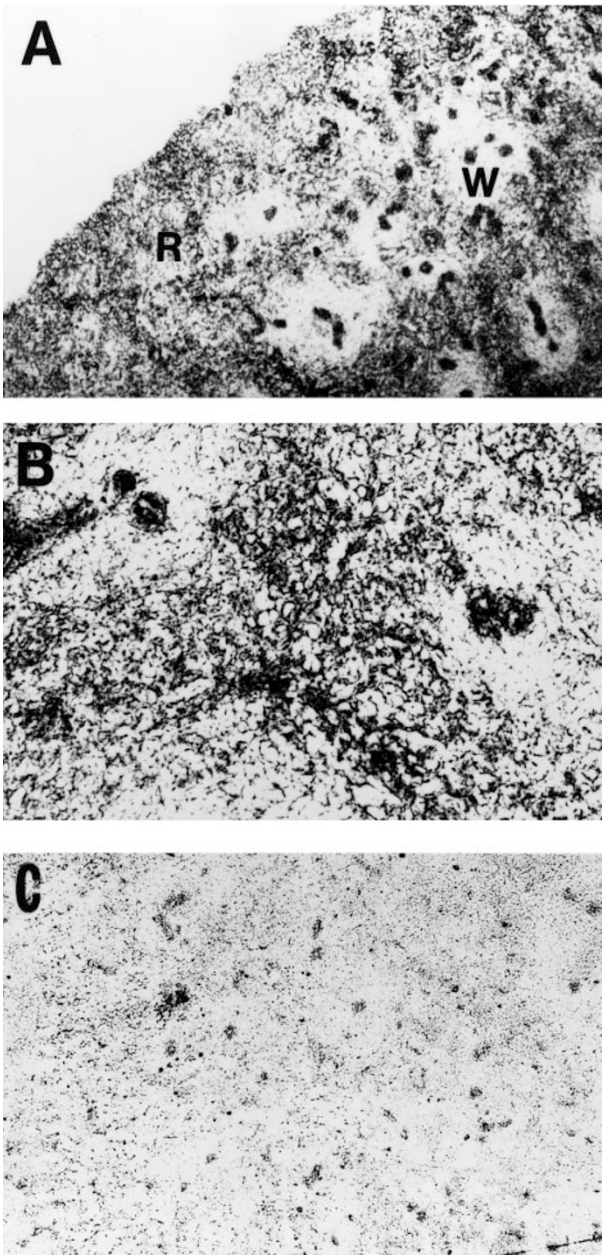

Figure 4 Expression of LTβR in developing human spleen. Frozen sections of spleen from a 20 week gestation fetus were stained for LTβR expression (black). (**A**) 145 × and (**B**) 580 ×. (**C**) Section stained with affinity purified control goat antiserum (145 ×). Red pulp (R) and white pulp (W) are indicated

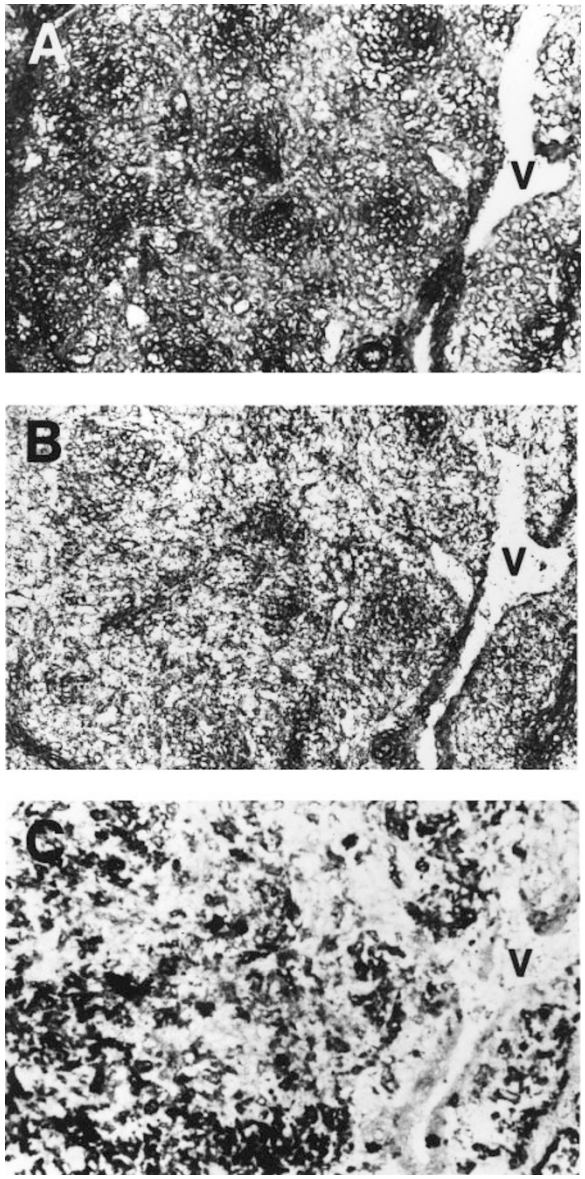

Figure 5 Colocalization of LTβR and reticular dendritic cells in human spleen. Serial sections of frozen spleen from 17 week gestation fetus were stained for (**A**) reticular dendritic cells, (**B**) LTβR or (**C**) CD11c positive tissue macrophages. Positive staining is indicated by black. All sections are at 580 ×. A large blood vessel (V) in all sections is indicated

Expression of LTβR and other TNFR family members on FDC-1

Our findings that LTβOR is expressed on stromal cells rather than lymphocytes in lymphoid tissues is consistent with the results shown in Table 1 and suggests that non-hematopoietic cells such as reticular dendritic cells express this receptor. To verify this hypothesis we used immunofluorescence and flow cytometry to examine expression of LTβR and other TNFR family members on a human follicular dendritic cell line, FDC-1, which is representative of this lineage of cells (Figure 6). As previously described (Clark *et al*, 1992), this line expresses CD40. These cells also express relatively high levels of LTβR, Fas and TNFR60, while staining with antibody to TNFR80 was undetectable on FDC-1 when compared to normal IgG control.

Ligation of LTβR with LTα1β2 enhances ICAM-1 (CD54) expression on FDC-1

To verify that the expression of LTβR on FDC-1 has functional significance, we cultured FDC-1 in the presence of TNF or a soluble form of LTα1β2 and examined the expression of

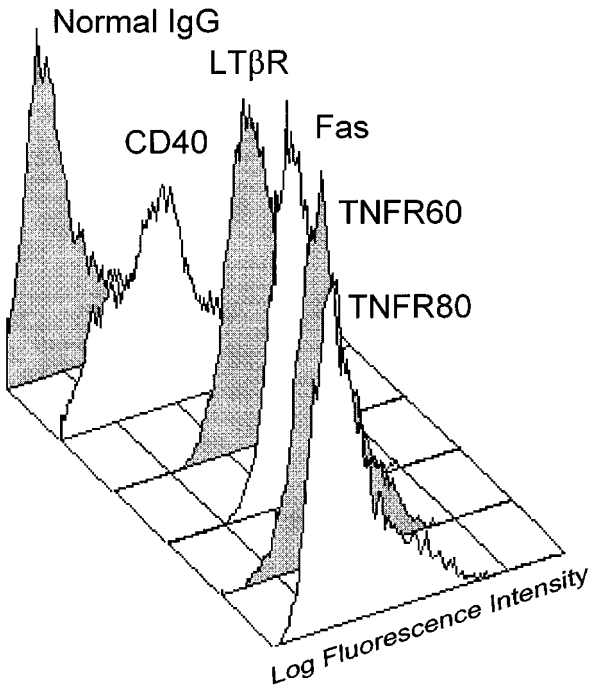

Figure 6 Expression of LTβR and other TNFR family members on FDC-1. FDC-1 cells were harvested by treatment with 20 mM EDTA in saline and stained with the indicated antibodies and PE-labeled $F(ab')_2$ goat-anti-mouse Ig. The mean fluorescence channel for: normal mouse IgG, 6; anti-TNFR60, 15; anti-TNFR80, 4; anti-LTβR, 31; anti-CD40, 13 and anti-Fas, 31

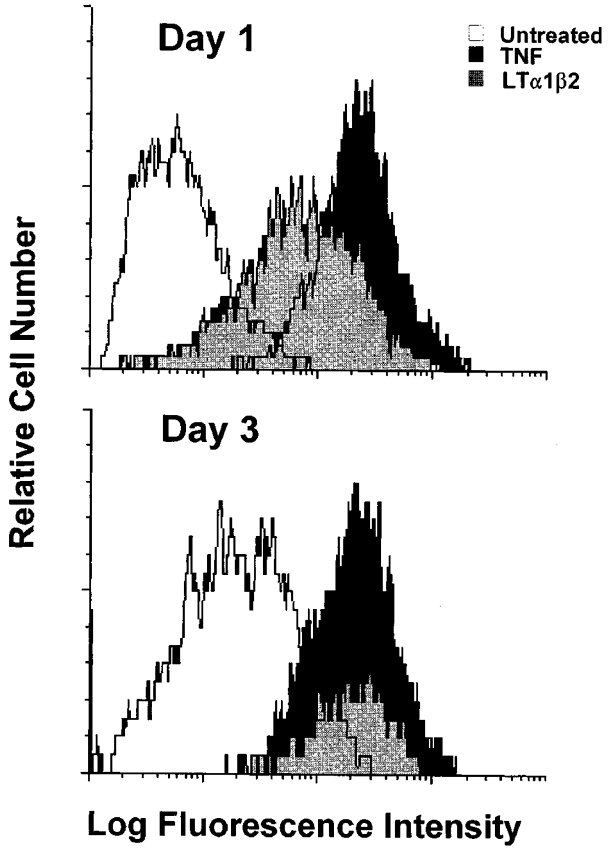

Figure 7 Enhancement of ICAM-1 expression by ligation of LTβR on FDC-1. FDC-1 cells were treated with TNF (0.2 nM) or soluble LTα1β2 (1.0 nM) for 1 – 3 days and then harvested with 20 mM EDTA/saline and stained with anti-CD54 (ICAM-1) and PE-labeled $F(ab')_2$ goat-anti-mouse Ig

ICAM-1 by immunofluorescence and flow cytometry (Figure 7). As described (Clark *et al*, 1992), these cells constitutively express low levels of ICAM-1, which is markedly induced by TNF. We observed that soluble LTα1β2 also induced ICAM-1 on these cells after only 1 day in culture and that this level was enhanced to that found with TNF by 3 days in culture. As LTβR is the only known receptor for LTα1β2 (Crowe *et al*, 1994; Browning *et al*, 1993), these data suggest that LTβR ligation results in a functional signal on this cell line.

Discussion

Characterization of LTβR expression in primary human peripheral blood cells and cell lines indicate that this receptor is not expressed on T or B lymphocytes and has a pattern of expression which is different from that of CD40, fas, TNFR60 and TNFR80. LTβR is also not expressed on resting, peripheral blood derived monocytes or DC (Figure 1). The fact that it was found at low levels on several myeloid cell lines (Table 1) suggests that LTβR may be present on immature or activated cells of the myeloid lineage. The LTα1β2 ligand for LTβR exists in a cell surface form which is expressed predominantly by activated T cells, but also by activated B cells and NK cells (Crowe *et al*, 1994; Androlewicz *et al*, 1992; Browning *et al*, 1991, 1993; Ware *et al*, 1992), suggesting a more restricted function for this receptor through direct cellular interaction with T cells. The fact that LTβR is expressed on stromal cells in lynphoid organs, sites where cellular interaction with T cells is likely to occur, strengthens this hypothesis.

Our finding of LTβR expression on stromal cells in human thymus is consistent with a previous report of LTβR mRNA expression in murine thymus and in a medullary thymic epithelial cell line (Force *et al*, 1996). Thus far, activated mature T cells have been shown to express LTα1β2. However, mRNA for LTα and LTβ have been detected in thymus (Browning *et al*, 1993), suggesting that immature thymocytes may also express this ligand. Despite these expression patterns, no obvious thymic abnormalities were reported in LTα or LTβ deficient mice or in mice treated with soluble LTβR : Fc fusion protein. It is possible that LTβR binds an alternative ligand in developing human thymus or that other cytokines are involved in thymic organogenesis.

Our observation of LTβR expression on reticular dendritic cells in the developing human spleen is consistent with the findings that mice transgenic for LTα under the control of the rat insulin promoter develop an accumulation of lymphocytes in the pancreas which strongly resembles *de novo* lymphoid organogenesis

(Kratz *et al*, 1996). Reticular dendritic cells are present in the red pulp and along the marginal areas which form a barrier between the red pulp and white pulp through which lymphocytes traverse (Weiss, 1972). As LTβR expression is most prominent in the red pulp it is important to note that the organization of the lymphoid elements within the spleen may involve signals delivered not only by lymphocytes, but also by stromal cells such as reticular dendritic cells. The LTα1β2 ligand exists as a cell surface protein on activated lymphocytes and is not secreted (Browning *et al*, 1991; Ware *et al*, 1992), whereas LTβR is present on reticular stromal cells, suggesting that this ligand/receptor interaction is through direct cellular contact between these two cell types.

FDC are believed to be derived from reticular stromal cells (Yoshida *et al*, 1995) which accumulate in the lymphoid follicles upon antigen stimulation and play an important role in B cell maturation in lymphoid tissues (Rajewsky *et al*, 1987; Szakal *et al*, 1989; Clark and Ledbetter, 1994). The importance of T and B cell interactions in lymphoid follicles has also been recently emphasized by the involvement of CD40 and CD40 ligand (Clark and Ledbetter, 1994), also members of the TNFR/TNF family. The expression of LTβR on the FDC-1 cell line, as well as our finding that ligation of this receptor results in induction of ICAM-1 on these cells, suggests that the LTα1β2 ligand on activated T or B cells, also found in lymphoid follicles (Rajewsky *et al*, 1987; Szakal *et al*, 1989; Clark and Ledbetter, 1994), interacts with LTβR on follicular stromal cells to influence the B cell maturation process. This hypothesis is supported by the recent finding that reconstitution of normal mice with bone marrow from LTα deficient mice results in loss of FDC clustering, germinal center formation and Ig class switching (Fu *et al*, 1997). Interestingly, mice deficient in TNF (Pasparakis *et al*, 1997) and TNFR60 (Matsumoto *et al*, 1996a,b; Pasparakis *et al*, 1997) also show failure of germinal center formation and FDC clustering with less dramatic effects on lymphoid organogenesis (Pasparakis *et al*, 1997). This result is consistent with our finding of TNFR60 on the FDC-1 cell line and suggests that both LTβR and TNFR60 signaling are important in FDC clustering and germinal center formation. In constrast to LTα deficient mice, LTβ knockout mice retain cervical and mesenteric lymphnodes and the disorganization of T and B cells in the spleen is not as severe (Koni *et al*, 1997; Alimzhanov *et al*, 1997) suggesting that LTα may engage another receptor.

In summary, our findings demonstrate that LTβR is present in human thymus and spleen at early stages of development and is localized to reticular dendritic cells, and not lymphocytes. The early expression and localization of this receptor on stromal cells implies that the interaction with cell surface LTα1β2 may be important in organization of these tissues. Additionally, our finding that LTβR is expressed on FDC-1 helps to explain the abnormalities in FDC clusters and antibody production observed in LTα and LTβ deficient mice and suggests that the LTα1β2/LTβR interaction plays an important role in lymphocyte interactions with FDC in the germinal center.

Materials and Methods

Antibodies

The following anti-human mAbs were used for flow cytometry: Anti-LTβR BDA8 mouse IgG1 biotin-conjugated and unconjugated forms (Browning *et al*, 1996b), anti-TNFR80 M1 rat IgG2b, (Ware *et al*, 1991), anti-TNFR60 H398 mouse IgG2b (Bender Med Systems, Vienna Austria), anti-CD40 G28-5 mouse IgGI (a gift from E. Clark, University of Washington, Seattle, WA), anti-Fas CH-11 mouse IgM (Pan Vera Inc, Madison, WI), anti-CD4 SK3 mouse IgG1 and anti-CD8 SKI mouse IgG1 FITC-conjugated (Becton Dickinson Immunocytochemistry Systems, San Jose, CA), anti-HLA-DR B8.11.2 mouse IgG2a biotin-conjugated (provided by J. van de Winkel, Utrecht, The Netherlands), anti-CD14 AML-223 mouse IgG2b FITC-conjugated (Medarex, Inc, Annandale, NJ), anti-CD33 4D3 mouse IgG2b PE conjugated (Caltag Laboratories, Inc., San Francisco), anti-CD83 HB-15a mouse IgG2b biotin-conjugated (a gift from T. Tedder; Duke University Medical Center, Durham, NC), anti-CD54, ICAM-1, mouse IgG1 (Chemicon International, Temecula, CA). Isotype matched control mAb were obtained from Medarex. TRI-COLOR-labeled streptavidin, PE and FITC-labeled $F(ab')_2$ goat-anti-mouse Ig, and PE-streptavidin were obtained from Caltag.

For immunohistochemistry serum specific for human LTβR was collected from goats immunized with a human LTβR : Fc fusion protein (Crowe *et al*, 1994) and adsorbed with human IgG to remove human Fc reactivity as described (VanArsdale and Ware, 1994). The goat anti-LTβR has no crossreactivity with TNFR60, TNFR80 or Fas (CD95) as measured by competitive radiobinding assays, immunoprecipitation or flow cytometry. Purified serum was used at a 1 : 1000 dilution (approx. 10 μg/ml) for immunohistochemistry. Murine mAbs 12B1 (IgG2a, Immunotech, Inc., Westbrook, ME) and anti-gp150,95 (IgG2b, Becton Dickinson), reactive with reticular dentritic cells (Farace *et al*, 1986) and CD11c on tissue macrophages (Lanier *et al*, 1985; Myones *et al*, 1988), respectively, were used at 10 μg/ml. Pre-immune goat serum was similarly treated by passage over immobilized human IgG column and used at similar dilutions for controls. Isotype-matched IgG (Organon-Teknika, West Chester, PA) were used as negative controls for mouse mAbs. Purified recombinant TNF and LTα1β2 (Browning *et al*, 1996a) were provided by Biogen, Inc.

Cell lines

The human cell lines HUT-78, Molt4, U937, HL60, K562, WI-38, HEK293 and ME-180 described in Table 1 were purchased from the American Type Tissue Collection (ATCC, Rockville, MD). The B cell-2, -3 and BJAB lines were obtained from E. Kieff and G. Mosialos (Harvard University, Boston, MA). B cell-3 was derived from a B cell enriched fraction of human peripheral blood cells infected with Epstein-Barr virus (EBV) carried in the marmoset B95-8 cell line. BJAB is from an EBV negative Burkitt's lymphoma. The human T cell line Jurkat (clone E6-1) was obtained from the NIH AIDS Research and Reference Reagent Program. The human follicular dendritic cell line FDC-1 (Clark *et al*, 1992) was a gift from E. Clark (University of Washington, Seattle, WA).

Human peripheral blood T cells, monocytes and dendritic cells (DC)

PBMC from healthy volunteers were obtained by F/H density gradient centrifugation (Boyum, 1968). Activated T cells were prepared by culture of PBMC for 5–8 days in RPMI-1640 containing 10% FCS, anti-CD3 (OKT3, 10 ng/ml) and, after 3 days, with IL-2 (10 ng/ml) as

described (Ware *et al*., 1992). For experiments in Table 1 these activated T cells were distinguished as CD4 or CD8 by immunofluorescence and flow cytometry using FITC-labeled anti-CD4 or CD8 and analyzed for expression of TNFRs with biotinylated mAbs and PE-streptavidin.

Blood dendritic cells (DC) and monocytes were enriched using countercurrent elutriation as previously described (Fanger *et al*, 1996). Briefly, PBMC were loaded onto the JE-6B elutriator (Beckman, Palo Alto, CA) and 50 ml fractions were collected while increasing the flow rate from 30 to 45 ml/min at 2500 r.p.m. Elutriated fractions consisting of fewer than 10% lymphocytes were obtained using this procedure and monocytes (CD33+, CD14+) and DC (CD33+, CD14$-$) could be distinguished. For experiments described enriched monocyte/DC fractions were analyzed for expression of CD33, CD14 and either CD83, HLA-DR or LTβR by three-color immunofluorescence and flow cytometry immediately (day 0) or following 2 days of culture in HEPES RPMI 1640 (Gibco, Grand Island, NY) containing 10% pooled human serum at 5×10^5 cells per well in a 96 well plate.

Human thymus and spleen

Postnatal thymus was obtained with the assistance of pediatric cardiothoracic surgeons at the University of California Medical Center (San Francisco, CA). Specimens of postnatal thymus from four normal infants ranging in age from 2 days to 22 months and two Down syndrome (DS, trisomy 21) infants aged 4 months and 19 months were examined. Fetal thymus and spleen were obtained from Advanced Bioscience Resources, Inc. (Alameda, CA). Specimens of thymus and spleen from 14, 16, 18, 20 and 22 weeks gestation were examined. Tissue was snap-frozen and stored at -70°C. Frozen sections (8 μm) of tissue were prepared and fixed in acetone at -20°C for 20 min on *Superfrost* slides (Fisher Scientific, Pittsburgh, PA).

Immunohistochemistry

Immunohistochemistry for human tissues was performed on acetone fixed frozen sections using Vector ABC *Elite* reagents (Vector Laboratories, Inc., Burlingame, CA). Modifications included the use of 3% blocking rabbit serum, dilution of antibodies in blocking serum, quenching of endogenous peroxidase in 0.3% H_20_2 in MeOH following reactivity with biotinylated secondary antibody and detection of specific peroxidase activity using Nickel-DAB (Pierce, Rockford, IL) and DAB Enhancing Solution (Vector Laboratories). Where indicated, sections were counterstained with 0.1% methyl green.

Immunofluorescence and flow cytometry

Cell analysis was performed on a FACScan (Becton Dickinson, San Jose, CA) with more than 5000 cells analyzed per sample. For single color immunofluorescence, cell suspensions (5×10^5 cells in 20 μl) were incubated for 1 h at 4°C with 20 μl of mAb at optimal concentrations. When necessary, 20 μl human IgG (12 mg/ml, Sigma, St. Louis, MO) was added to block Fc receptor binding. Following three washes with 200 μl PBS containing 2 mg/ml BSA, 40 μl of PE-labeled F(ab′)2 goat-anti-mouse Ig (20 μg/ml) was added for an additional 1 h. For dual color immunofluorescence with anti-CD4 or anti-CD8 FITC, biotin-conjugated primary mAbs and PE-streptavidin were used. Cells were washed three times, resuspended in PBS-BSA for immediate analysis or PBS-BSA supplemented with 1% paraformaldehyde for analysis 1 to 7 days later. For three-color analysis cells were incubated with biotinylated primary antibody, washed as above, then followed with TRI-COLOR Streptavidin, PE-anti-CD33 and FITC-anti-CD 14, washed and fixed as above.

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