to about 28.8 cM with the use of the Kosambi mapping function [D. D. Kosambi, *Ann. Eugen.* **12**, 172 (1944)]. MAPMAKER [E. S. Lander *et al.*, *Genomics* **1**, 185 (1987); modified by S. Tingey for the Macintosh] allows use of either the Haldane [J. B. S. Haldane, *J. Genet.* **8**, 299 (1919)] or the Kosambi mapping function. Because the Kosambi function assumes a greater amount of positive interference [which occurs in zebrafish (*3*)], it was selected. For analysis in MAPMAKER, markers in repulsion were inverted. MAPMAKER provided scores of the logarithm of the likelihood ratio for linkage (lod scores; all linkages in this map were supported by a lod score of >3) and converted the frequency of recombinants into map distance in centimorgans.

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34. To detect potential errors, two people scored gels independently. Markers with discrepancies greater than 5% were excluded from analysis. In other cases, discrepancies were resolved by reexamination of the original photograph or by reamplification. Duplicate reactions of some primers involving nearly 1000 data points indicated scoring error (because of incorrect amplification) of about 1% or less. After linkage groups were ordered with MAPMAKER, double recombinants in short (<10 cM) intervals [an indication of error in the data set; W. Dietrich *et al.*, *Genetics* **131**, 423 (1992)] were identified and rechecked against original gel pictures or by new reactions. There were 42 such doubles remaining out of about 39,000 data points [94 haploids × (401 RAPDs + 13 SSRs + 3 cloned genes)]. These may reflect error or may be bona fide recombination events. Markers with a high error rate often tend to map spuriously to the ends of LGs because that location causes errors to appear as single crossovers rather than as double crossovers. In our data set, terminal intervals were on average a bit longer (8.7 ± 7.6 cM SD) than the average interval on the map (5.8 ± 5.9 cM), but the difference was not statistically significant. A total of 48 markers (about 12% of the total) on 10 LGs were retested for LG assignment in a number of crosses in different genetic backgrounds. The location of all markers tested was confirmed, indicating the robustness of LG assignments.
35. S. L. Johnson, unpublished results.
36. This paper is dedicated to C. Walker for pioneering and continuing work in zebrafish genetics. We acknowledge support from the following: NIH grant 1RO1AI26734, Medical Research Fund of Oregon, and American Heart Association Oregon Affiliate (J.H.P.); NIH grant HD07470 (S.L.J.); NIH grant NS23915 and Research Career Development Award grant NS01476 (J.S.E.); and the University of Oregon Zebrafish Program Project grant 1PO1HD22486 (J. Weston). W.S.T. is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research. We thank R. Sederoff and R. Lande for discussions; M. Halpern, E. Selker, G. Sprague, and M. Westerfield for helpful comments on the manuscript; and W. Potts for the MHC primers. Linker and the mapping stocks are available (AB from C.B.K. and a partially inbred Darjeeling stock from S.L.J.).

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Abnormal Development of Peripheral Lymphoid Organs in Mice Deficient in Lymphotoxin

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Mice rendered deficient in lymphotoxin (LT) by gene targeting in embryonic stem cells have no morphologically detectable lymph nodes or Peyer's patches, although development of the thymus appears normal. Within the white pulp of the spleen, there is failure of normal segregation of B and T cells. Spleen and peripheral blood contain $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells in a normal ratio, and both T cell subsets have an apparently normal lytic function. Lymphocytes positive for immunoglobulin M are present in increased numbers in both the spleen and peripheral blood. These data suggest an essential role for LT in the normal development of peripheral lymphoid organs.

Lymphotoxin (LT, also designated TNF-β) is a soluble product of activated lymphocytes that was first defined by its cytotoxic activity against fibroblasts (*1–3*). LT is now recognized to be produced by activated $CD4^+$ T helper cell type 1 (T_H1) lymphocytes, $CD8^+$ lymphocytes, and certain B lymphoblastoid and monocytoid cell lines (*4–6*). The gene encoding murine LT is located 1100 base pairs (bp) upstream of the evolutionarily related gene encoding tumor necrosis factor–α (TNF-α) within the major histocompatibility complex (MHC) (*7, 8*). The LT and TNF-α proteins are structurally related and show similar activities in vitro and when given to experimental animals (*9*). In solution, LT is a homotrimer with a conformation similar to that of TNF-α. A membrane-associated form of LT has been described, consisting of a heterotrimeric complex containing two LT monomers together with a 33-kD transmembrane protein designated LT-β (*10*). The gene encoding LT-β is located immediately centromeric to the gene encoding TNF-α. The biological effects of LT and TNF-α are mediated by two receptors, designated p55 and p75 (*11*).

In vitro, LT and TNF-α can modulate many immune and inflammatory functions. As implied by their names, both cytokines are cytotoxic for a variety of transformed and normal cell types (*12–14*). In order to be killed, target cells must express LT–TNF-α receptors, with the p55 receptor appearing to mediate the cytotoxic response (*15*). LT and TNF-α can augment the proliferation of activated thymocytes (*16*)

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and B cells (*17*). They also induce the expression of MHC class I and class II antigens in many cell types, including endothelial and pancreatic islet cells (*18, 19*). TNF-α and LT also appear to induce adhesion of leukocytes to vascular endothelia by activating the expression of adhesion proteins on the endothelial cells (*20*). Because LT and TNF-α bind to the same cell surface receptors and in vitro activate the same cellular responses, it has been suggested that in vivo they may be in large part functionally redundant. In this model, specificity of LT and TNF-α function could derive from selective activation of the gene encoding LT primarily in activated lymphocytes and from activation of the gene encoding TNF-α more broadly in mononuclear phagocytes, activated lymphocytes, and many other cell lineages.

Unique activities of LT in immune homeostasis have not, however, been defined. Progress in defining the natural functions of LT has been impeded by the absence of LT-specific antibodies and bioassays in the principal animal model, the mouse. All available LT and TNF-α polyclonal and monoclonal antibodies appear to detect both mouse cytokines. Furthermore, currently available bioassays use cells expressing either the p55 or p75 TNF receptor, which bind and respond to both LT and TNF-α. Our study here was undertaken to identify natural functions of LT by production of an LT-deficient mouse strain with the use of gene targeting in murine embryonic stem (ES) cells (*21*).

We constructed a targeting vector by replacing a small portion of the second exon, the entire third exon, and a small portion of the fourth exon of the gene encoding murine LT with a *neo*^{r} cassette (Fig. 1B). The *neo*^{r} gene disrupts the LT coding sequence and introduces a termination codon after LT exon 2. Homologous recombination in ES cells introduces new Pst I and Hind III sites from the *neo*^{r} gene into the LT locus. These sites can be identified by Southern (DNA) blotting with an LT–TNF-α intergenic probe (Fig. 1, A and B). Three independent targeted ES lines were used for blastocyst injection, and one line yielded germline-transmitting chimeras. Heterozygotes for the targeted allele were mated, and $LT^{-/-}$, $LT^{-/+}$, and $LT^{+/+}$ offspring were obtained at the expected frequencies, which indicates that homozygous LT deficiency is not associated with abnormal fetal loss. $LT^{-/-}$ and $LT^{-/+}$ mice were observed through 6 months of age and appeared grossly normal compared to their wild-type littermates. Specifically, they showed no evidence of acute or chronic infection. For analysis of cytokine expression and lymphoid development, mice were studied between 5 and 8 weeks of age.

Spleen cells from $LT^{+/+}$ mice showed predominant 1.5-kb and 1.8-kb LT transcripts and a minor 3.1-kb transcript present in small amounts 4 hours after treatment with concanavalin A (Con A) and in greater amounts after 24 hours (Fig. 2). These are the usual sizes of the various processed forms of LT mRNA (*22*). In $LT^{-/-}$ cells, the 1.5- and 1.8-kb transcripts were absent and a major 3-kb transcript was observed. The size of this transcript is consistent with the structure of the targeted gene, with the 1.6-kb *neo*^{r} fragment interposed between the second and fourth exons of the LT locus (*21*). Transcripts from the targeted gene encoding LT are predicted to encode 31 residues of the 33–amino acid LT signal peptide followed by 91 residues derived from the antisense *neo*^{r} sequence. Hybridization with a *neo*^{r} gene probe demonstrated the same 3.0-kb fragment as well as the anticipated 1.6-kb sense *neo*^{r} transcript. TNF-α transcripts were detected in spleen cells 4 hours after treatment with Con A (Fig. 2), returning to near base line by 24 hours. The quantities of TNF-α transcripts were similar in cells from $LT^{-/-}$ and $LT^{+/+}$ animals. A bioassay that detects both LT and TNF-α was performed with supernatants of spleen cells stimulated with Con A. At 4 hours, there were 128 units of cytotoxic activity from $LT^{+/+}$ spleen cells and 32 units from $LT^{-/-}$ spleen cells (*23*). These data are consistent with the presence of TNF-α and LT in supernatants of Con A–stimulated $LT^{+/+}$ spleen cells and the presence of TNF-α activity without LT in supernatants of spleen cells from $LT^{-/-}$ animals.

$LT^{-/-}$ mice showed abnormal development of peripheral lymphoid organs. $LT^{-/-}$ mice showed no detectable popliteal, inguinal, para-aortic, mesenteric, axillary, or cervical lymph nodes (Fig. 3). Popliteal lymph nodes in $LT^{+/+}$ and $LT^{-/+}$ mice (Fig. 3) were visualized directly after injection of India ink into the footpad and by histologic analysis. In $LT^{-/-}$ mice, neither lymphoid aggregates nor rudiments of

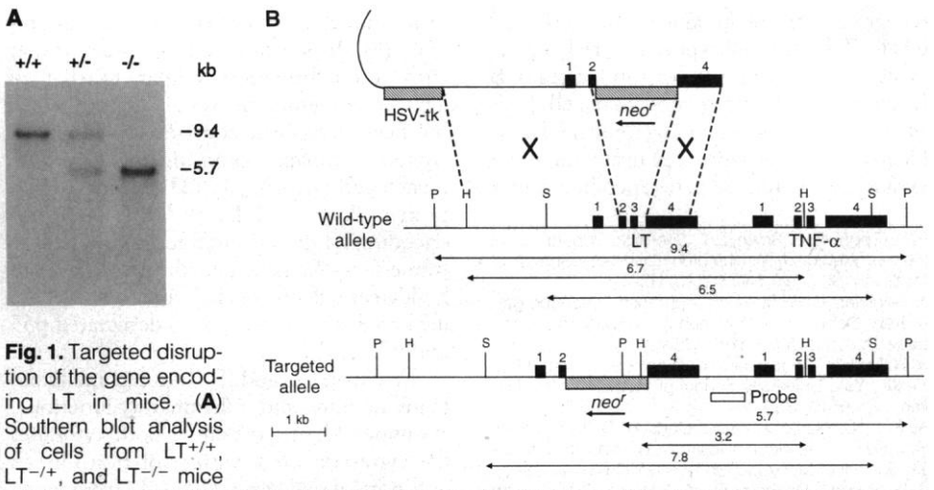

Fig. 1. Targeted disruption of the gene encoding LT in mice. (**A**) Southern blot analysis of cells from $LT^{+/+}$, $LT^{-/+}$, and $LT^{-/-}$ mice with Pst I and the 634-bp Nco I fragment probe. (**B**) Structure of the targeting vector, the gene encoding wild-type mouse LT, and the predicted targeted LT allele. Black boxes indicate exons of the genes encoding LT and TNF-α. Gene targeting in ES cells results in acquisition of new Pst I and Hind III sites, as indicated. H, Hind III; P, Pst I; and S, Ssp I. The location of the TNF-α–LT intergenic Nco I fragment probe is indicated. The lengths of the indicated restriction fragments are in kilobases.

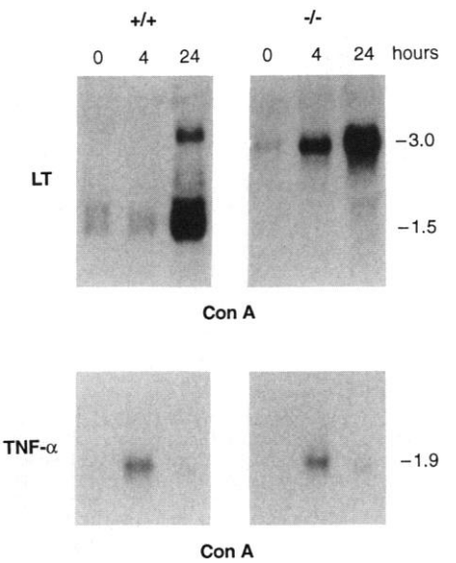

Fig. 2. Northern (RNA) blot analysis of Con A–stimulated spleen cells from $LT^{+/+}$ (left) and $LT^{-/-}$ (right) mice. Cells were cultured at a concentration of 10^7 cells/ml with Con A (5 μg/ml). Cells (10^8) were harvested instantly and 4 and 24 hours later. Hybridization probes were ^{32}P-labeled fragments of the LT and TNF-α cDNA [LT, 1.3-kb Hinc II fragment of pM7A (*12*); TNF-α, 1.1-kb Eco RI–Pst I fragment of pMuCachectin]. Sizes are indicated on the right in kilobases.

lymph node stromal tissue were detected either by gross inspection or by histologic examination. Intradermal injection of complete Freund's adjuvant around the base of the tail did not lead to the development of detectable nodes in the $LT^{-/-}$ mice, and they also showed no grossly detectable Peyer's patches.

Morphological changes were also seen in the spleens of $LT^{-/-}$ mice. Total numbers of nucleated spleen cells were similar in 6-week-old $LT^{-/-}$ and $LT^{+/+}$ mice (0.8×10^8 to 1.2×10^8 cells per spleen; $n = 5$); however, in $LT^{-/-}$ mice there were alterations in T and B lymphocyte content and in splenic architecture (Fig. 4). Spleens of $LT^{-/-}$ mice contained slightly reduced numbers of T lymphocytes (*24*), with 3.1×10^7 T cells per spleen in $LT^{+/+}$ animals and 2.6×10^7 T cells per spleen in $LT^{-/-}$ animals, and a similar ratio of $CD4^+CD8^-$ and $CD4^-CD8^+$ cells (2.0 in $LT^{+/+}$ animals and 2.5 in $LT^{-/-}$ animals). There was a 30% increase in the number of immunoglobulin M–positive and B220-positive (IgM^+B220^+) B lymphocytes in the spleens of $LT^{-/-}$ animals compared to that in $LT^{+/+}$ mice. By histological analysis, the spleens of $LT^{-/-}$ mice showed changes in the white pulp (Fig. 4, A and B), with altered organization of the periarteriolar T cell zone and loss of a distinct marginal zone. Localization of T and B cells with immunofluorescence microscopy showed normal segregation of B cells and T cells within the white pulp in the spleens of $LT^{+/+}$ mice, with T cells clustered in the periarteriolar region (Fig. 4C) and B cells peripheral to the T cell zone (Fig. 4E). Thy-1–staining cells in the spleens of $LT^{-/-}$ mice were scattered throughout the white pulp, with clustering in the periarteriolar region (Fig. 4D). In contrast to the distribution in wild-type animals, IgM^+ cells were present throughout the white pulp (Fig. 4F), including the periarteriolar region.

The peripheral blood of $LT^{-/-}$ mice showed normal numbers of $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells. There was, however, a nearly fourfold increase in B cells in the $LT^{-/-}$ animals. The total white blood cell count was 4000 mm^{-3} in $LT^{+/+}$ mice and 11,500 mm^{-3} in $LT^{-/-}$ mice ($n = 3$). In $LT^{-/-}$ mice, the neutrophil, monocyte, and platelet counts were normal. The thymuses in $LT^{-/-}$ mice showed apparently normal medullary and cortical zones (Fig. 4, G and H) and contained normal numbers of $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ cells. Intraepithelial lymphocytes were present with apparently normal distribution in the small intestine, which suggests that LT is not required for localization of γδ T cells.

Splenic T cells from $LT^{-/-}$ mice were able to develop brisk MHC class I– and class II–restricted allocytotoxic responses (*25*). After in vitro stimulation, activated mixed T cell populations from $LT^{+/+}$ and $LT^{-/-}$ mice showed similar killing of P815 (MHC class I^+, class II^-) and TA3 (MHC class I^+, class II^+) cells (Fig. 5, A and B). Purified activated $CD4^+$ cells from $LT^{-/-}$ mice also showed similar cytotoxicity compared to those in $LT^{+/+}$ mice (Fig. 5, C and D). Under conditions in which cells from $LT^{-/-}$ and $LT^{+/+}$ mice efficiently killed

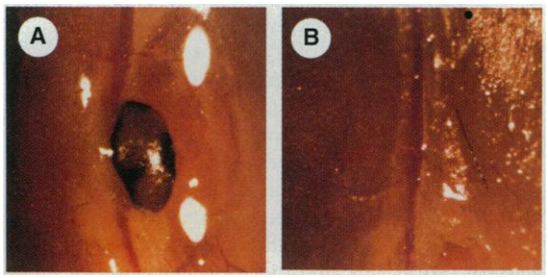

Fig. 3. Absence of visible lymph nodes in $LT^{-/-}$ mice. India ink (0.02 ml) was injected into the hind footpads of 6-week-old $LT^{-/+}$ and $LT^{-/-}$ mice. One and a half hours later, the animals were killed, and their popliteal fossae were dissected. (**A**) Visualization of lymph nodes in the $LT^{-/+}$ mice was facilitated by their content of India ink. (**B**) The popliteal fossae of $LT^{-/-}$ mice showed no detectable lymphoid tissue and no India ink staining when observed under a dissecting microscope.

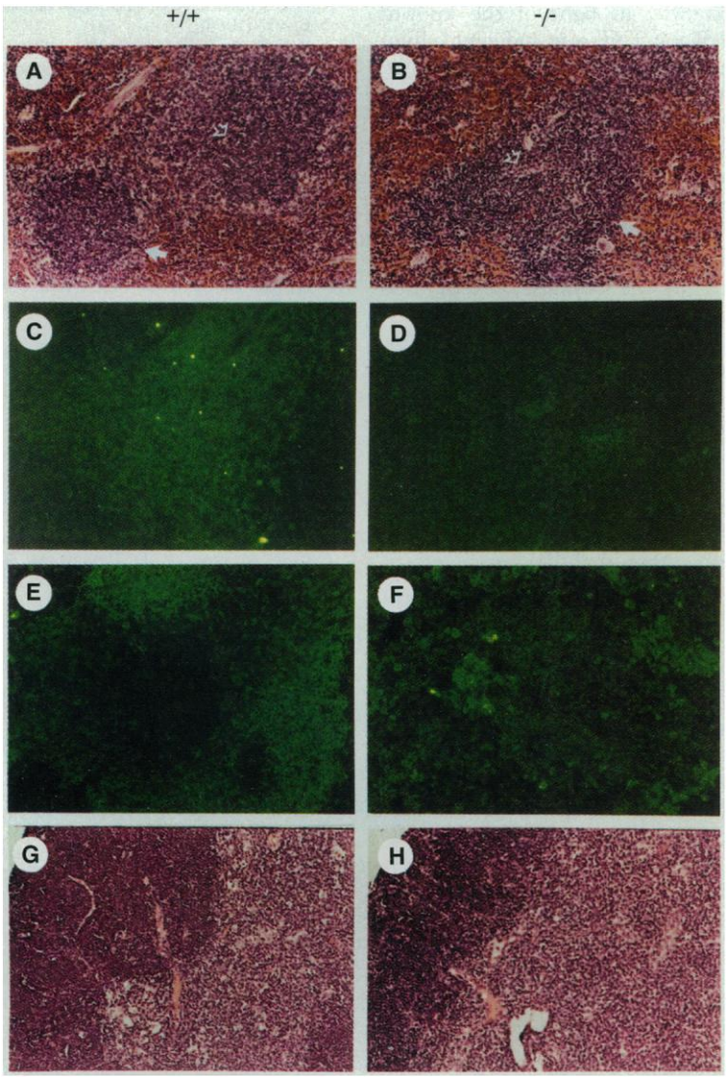

Fig. 4. Organization of lymphoid cells in the spleens and thymuses of $LT^{-/-}$ mice. The left column shows results for $LT^{+/+}$ mice; the right column shows results for $LT^{-/-}$ mice. (**A** and **B**) Spleen cells stained with hematoxylin-eosin. Solid arrows indicate the locations of the marginal zone. Open arrows indicate the locations of the periarteriolar sheath. (**C** and **D**) Spleen cells, shown here with immunofluorescence with antibodies to Thy-1. (**E** and **F**) Spleen cells, shown here with immunofluorescence with antibodies to IgM. (**G** and **H**) Thymus cells, shown here with hematoxylin-eosin staining. (C) through (F) are from serial sections. Magnification, ×200.

P815 targets (Fig. 5E), these same cells showed similar induction (Fig. 5F) of the nuclear lesion that is characteristic of apoptosis (*26*). These data demonstrate no substantial defect in either CD8- or CD4-dependent cytotoxic function in $LT^{-/-}$ mice, which suggests that LT is not essential for these in vitro cytotoxic activities. In preliminary experiments, the proliferative response of splenic T cells was assessed after immunization with *Escherichia coli* β-galactosidase in complete Freund's adjuvant. Similar proliferative responses were seen in $LT^{+/+}$ and $LT^{-/-}$ mice, which suggests that $LT^{-/-}$ T cells can respond to antigen challenge. Because $LT^{-/-}$ mice have a compound phenotype (deficiency of LT expression and also abnormal structure of peripheral lymphoid organs), it will be important ultimately to test the consequences of LT deficiency in the context of a normal lymphoid architecture, perhaps in normal irradiated mice whose bone marrow has been reconstituted with bone marrow from an $LT^{-/-}$ mouse.

Our data demonstrate fundamental actions of LT in lymphoid organogenesis. A role for the LT–TNF-α axis in embryonic development has been suggested by studies

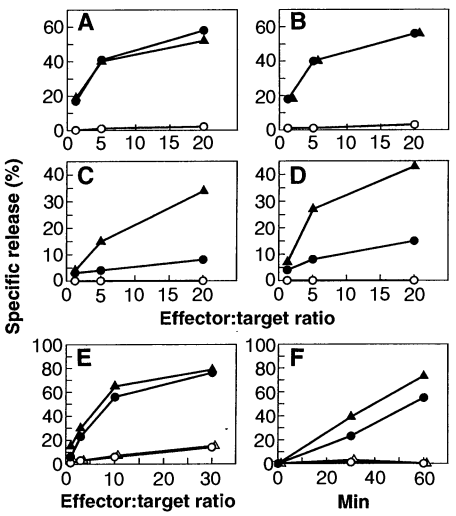

Fig. 5. Target cell killing by $LT^{-/-}$ lymphocytes. Cytolytic effector cells were prepared from $LT^{+/+}$ or $LT^{-/-}$ splenocytes as described (*25*). (A) through (E) show ^{51}Cr release; (F) shows ^{125}I-UdR release. In (A) through (D) there was a 6-hour incubation; closed circles, P815; closed triangles, TA3; open circles, EL4. (**A**) $LT^{+/+}$ T cells (15% $CD4^+$, 79% $CD8^+$). (**B**) $LT^{-/-}$ T cells (17% $CD4^+$, 72% $CD8^+$). (**C**) $LT^{+/+}$ $CD4^+$ cells (82% $CD4^+$, 0% $CD8^+$). (**D**) $LT^{-/-}$ $CD4^+$ cells (75% $CD4^+$, 0% $CD8^+$). In (**E**) and (**F**) is shown the target cell killing with spleen effector T cells (circles, $LT^{+/+}$: 12% $CD4^+$ and 70% $CD8^+$; triangles, $LT^{-/-}$: 15% $CD4^+$ and 63% $CD8^+$); closed symbols, P815 targets; open symbols, EL-4 targets. (E) shows ^{51}Cr release at 4 hours; (F) shows ^{125}I-UdR release at 30 and 60 min with an effector:target ratio of 10:1.

that demonstrate LT–TNF-α cytotoxic activity or TNF-α mRNA in mouse embryos (*27*) and in human placenta and amniotic fluid (*28, 29*). TNF-α has also been detected in human fetal thymus (*30*) and in murine thymus (*31*). The participation of LT or TNF-α in early postnatal development has been suggested by studies (*32*) in which administration of a rabbit antiserum to murine TNF, either during pregnancy or at birth, leads to transient growth retardation and atrophy of the thymus, spleen, and lymph nodes; however, subsequent studies that used a chimeric protein made up of the p55 chain of the TNF receptor and the heavy chain of IgG to inhibit LT–TNF-α activity showed no alterations of lymphoid development (*33*).

The mechanism by which LT contributes to normal lymphoid development remains unknown. In light of the known action of LT and TNF-α in inducing adhesion of leukocytes to endothelia (*20*), a requirement for LT in an essential interaction between bone marrow–derived lymphoid cells and lymphoid stromal cells seems likely. The action of LT in this interaction apparently does not require the p55 TNF receptor, because mice deficient in this receptor show normal numbers of lymphocytes in the thymus, spleen, and lymph nodes (*34*). Thus, either the p75 receptor or other, as yet undefined, perhaps LT-specific receptors contribute to this effect. In this regard, the description of a p55-related receptor with specificity for an LT–LT-β heteromer (*35*) is of considerable interest. The fact that the $LT^{-/-}$ mice exhibit abnormal lymphoid development in the face of presumed normal TNF-α expression suggests either that LT has an effect that is not duplicated by TNF-α or that LT is expressed at a particular stage in development by a cell that is not expressing TNF-α. Although it is possible that absence of LT expression in T and B lymphocytes leads to the failure to develop normal partitioning of cells within the spleen, the existence of lymph node rudiments in nude (*nu/nu*) and SCID (severe combined immune deficient; *scid*) mice suggests that the failure to develop lymph nodes in $LT^{-/-}$ mice is due to the participation of cells other than B and T lymphocytes. Finally, the relation of the failure of lymphoid organ development in $LT^{-/-}$ mice to the recently described spontaneous mouse mutation, *aly* (*36*), merits attention. This autosomal recessive mutation results in the systemic absence of lymph nodes and Peyer's patches as well as the absence of distinct lymphoid follicles in the spleen in a fashion similar to the situation in $LT^{-/-}$ mice. Unlike $LT^{-/-}$ mice, mice with the *aly* mutation also have abnormal thymic architecture, failure in females to develop mammary glands, and a generalized deficiency of cell-mediated and humoral immunity. These differences establish that the $LT^{-/-}$ and *aly* mutations are not the same but suggest the existence of a group of genes that together control essential components of the development of peripheral lymphoid tissues.

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21. The genes encoding LT and TNF-α were isolated on a single clone from a BALB/c cosmid library [D. D. Chaplin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6947 (1983)], with pMuCachectin complementary DNA (cDNA) as the probe. A 4.5-kb Hind III–Xba I fragment containing the gene encoding LT was subcloned into pBluescript KS(+) (Stratagene) containing the gene encoding herpes simplex virus–thymidine kinase (HSV-tk) under the control of a polyoma promoter and enhancer [S. L. Mansour, K. R. Thomas, M. R. Capecchi, *Nature* **336**, 348 (1988)]. A 420-bp Apa I–Kpn I fragment containing three nucleotides of LT exon 2 through the first five nucleotides of LT exon 4 was replaced by a linker containing a Sal I site. A 1.6-kb Xho I fragment encoding neomycin resistance (*neo*^{r}) under the control of the pgk promoter [P. Soriano, C. Montgomery, R. Geske, A. Bradley, *Cell* **64**, 693 (1991)] was inserted into the Sal I site in the opposite transcriptional orientation compared to the gene encoding LT. The plasmid contains 2.5 kb that is homologous to LT genomic DNA 5′ of the *neo*^{r} gene and 0.88 kb (also homologous to LT genomic DNA) 3′ of the *neo*^{r} gene. It predicts a transcript from the LT promoter that contains the LT 5′ untranslated sequence and most of the LT signal peptide [93 nucleotides (nt) in exon 2], followed by 1660 nt of the reverse orientation *neo*^{r} fragment, 986 nt of the LT exon 4, and a polyadenylate [poly(A)] tail [total anticipated size is 2901 nt plus the poly(A) tail]. A termination codon is located 276 nt into the antisense *neo*^{r} fragment. Thus, the transcript predicts a polypeptide product of 31 residues of the LT signal peptide followed by 91 residues of antisense *neo*^{r} sequence only. The linearized vector was electroporated into the D3 ES line, and cells were plated on primary embryonic fibroblasts from day 14 embryos obtained by mating a male C1D mouse (GenPharm, International, Mountain View, CA) with a female Swiss Webster mouse (Taconic Farms, Germantown, NY). Selection was with 2 μM ganciclovir (Cytovene, Syntex Laboratories, Palo Alto, CA) and G418 (250 μg/ml;

Gibco-BRL). Double drug-resistant colonies were screened by Southern blotting with an LT–TNF-α intergenic probe. Homologous recombination was detected at a frequency of 1 in 63 drug-resistant colonies. Chimeric animals were obtained by injecting targeted cells into C57BL/6 blastocysts. Male mice showing >50% coat color chimerism were mated to C57BL/6 females, and germline transmission was documented by the presence of the 5.7-kb Pst I fragment.
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23. Bioactivity of TNF-α–LT was measured with the WEHI 164 fibrosarcoma cell line as described [N. H. Ruddle *et al.*, *J. Exp. Med.* 172, 1193 (1990)]. The specificity of the assay was defined with a neutralizing monoclonal antibody, TN3.19.12, that recognizes both murine TNF-α and LT.
24. Erythrocyte-depleted cells (10^6) were stained for 1 hour with fluoresceinated monoclonal antibodies to CD8 (Becton Dickinson), phycoerythrin-labeled monoclonal antibodies to CD4 (Becton Dickinson), fluoresceinated monoclonal antibodies to IgM (Pharmingen), phycoerythrin-labeled monoclonal antibodies to CD45R and B220 (Pharmingen), or biotinylated monoclonal antibodies to CD3ε (Pharmingen). The biotinylated monoclonal antibody was detected with streptavidin-RED613 (Gibco). Flow cytometric analysis was performed with the Lysis-I program (Becton Dickinson) on a FACScan (Becton Dickinson); data are means from experiments with three animals.
25. Cytolytic effector cells were established by primary, one-way mixed lymphocyte cultures of 1×10^7 to 2×10^7 $LT^{+/+}$ or $LT^{-/-}$ splenocytes ($H\text{-}2^b$) with 2×10^7 irradiated [absorbed dose of radiation (Gy) = 20 Gy] BALB/c ($H\text{-}2^d$) splenocytes. Responding cells were either unfractionated splenocytes, purified T cells (eluted from nylon wool then treated with monoclonal antibody J11d and complement), or purified $CD4^+$ T cells (eluted from nylon wool then treated with J11d and the CD8 monoclonal antibody 3.155 and complement). After culture for 5 to 6 days, live effector cells were isolated by Ficoll-Hypaque density gradient centrifugation. Target cells (0.5×10^6 to 1.0×10^6) were labeled in 1 ml of RPMI-1640 for 90 min with 500 μCi of $Na_2{}^{51}CrO_4$ or 10 μCi of 5′ ^{125}I-labeled iododeoxyuridine (^{125}I-UdR) as indicated, washed, incubated in the absence of label for 30 to 45 min, washed again, and added to varying numbers of effector cells. At the indicated times, release of label into the medium was assessed. After 6 hours, spontaneous ^{51}Cr release of P815 cells ($H\text{-}2^d$) was 7%, of TA3 cells ($H\text{-}2^d$) was 21%, and of EL-4 cells ($H\text{-}2^b$) was 5%. The nuclear lesion was assessed by termination of the incubation with 1 mM EDTA and 0.2% Triton X-100. Spontaneous release of ^{125}I-UdR was ≤14%.
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37. We gratefully acknowledge P. M. Allen, L. E. Fields, M. Peters, and E. R. Unanue for helpful discussions concerning murine lymphoid development and lymph node and spleen architecture; O. Kanagawa for discussion of *aly* mice; C. Bergman for LT-TNF bioassays; B. Beutler at the University of Texas, Southwestern Medical School, for pMuCachectin cDNA; T. Doetschman at the University of Cincinnati for the D3 ES cell line; and E. R. Unanue for evaluation of murine histopathology. Supported in part by NIH grants CA 16885 (N.H.R.) and CA 28533 (J.H.R.), and National Multiple Sclerosis Society grant RG-2394 (N.H.R.). D.D.C. is an investigator of the Howard Hughes Medical Institute.

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A Lymphotoxin-β–Specific Receptor

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Tumor necrosis factor (TNF) and lymphotoxin-α (LT-α) are members of a family of secreted and cell surface cytokines that participate in the regulation of immune and inflammatory responses. The cell surface form of LT-α is assembled during biosynthesis as a heteromeric complex with lymphotoxin-β (LT-β), a type II transmembrane protein that is another member of the TNF ligand family. Secreted LT-α is a homotrimer that binds to distinct TNF receptors of 60 and 80 kilodaltons; however, these receptors do not recognize the major cell surface LT-α–LT-β complex. A receptor specific for human LT-β was identified, which suggests that cell surface LT may have functions that are distinct from those of secreted LT-α.

The TNF ligand family encompasses a large group of secreted and cell surface proteins that may affect the regulation of inflammatory and immune responses (*1*). Secreted forms of TNF and lymphotoxin-α (LT-α, previously referred to as TNF-β) are homotrimers (*2*) that initiate similar spectra of cellular responses by binding to two specific cell surface receptors of 60 and 80 kD ($TNFR_{60}$ and $TNFR_{80}$) (*3*). Membrane TNF, a type II transmembrane protein, is the precursor of secreted TNF (*4*); in contrast, cell surface LT is structurally different from secreted LT-α and TNF, which suggests that it may not bind to $TNFR_{60}$ or $TNFR_{80}$. Cell surface LT is a heteromeric complex composed of LT-α and LT-β subunits (*5*). Lymphotoxin-β is a type II transmembrane member of the TNF ligand family that provides the membrane anchor for the attachment of the heteromeric complex to the cell surface (*6*). To test if LT-β confers distinct receptor binding properties to the cell surface LT-α–LT-β complex, we used soluble versions of the TNFRs [engineered as fusion proteins between the ligand binding domain of TNFR and the Fc region of immunoglobulin G1 (IgG1) (TNFR:Fc)] as probes for membrane-bound cytokines (*7*).

Cell surface LT and TNF were rapidly induced on the human $CD4^+$ T cell hybridoma II-23 by phorbol myristate acetate (PMA), as detected by fluorescence staining with specific monoclonal antibodies (mAbs) (Fig. 1A) (*8*). Membrane TNF expression peaked 3 to 4 hours after stimulation with PMA and declined rapidly. In contrast, cell surface LT expression was maximal 6 to 8 hours after stimulation and remained detectable for more than 24 hours (Fig. 1B). The $TNFR_{60}$:Fc and $TNFR_{80}$:Fc chimeric proteins had similar staining patterns on II-23 cells activated for 6 hours with PMA (Fig. 1C). To determine which cell surface ligands bind $TNFR_{60}$:Fc, we used LT-α mAb (9B9) or TNF mAb (104C) competitively for staining of PMA-activated II-23 cells. Both of these mAbs block binding of the soluble ligands to TNFRs and directly neutralize cytotoxic function (*9*). In the presence of saturating concentrations of competitor mAb, specific staining for $TNFR_{60}$:Fc was partially blocked with an LT-α mAb (Fig. 1D) and more efficiently with a TNF mAb (Fig. 1E), which indicates that membrane TNF binds to $TNFR_{60}$. The latter finding is consistent with reports that membrane TNF is cytotoxic (*4*); however, these results also suggest that a less abundant form of the cell surface LT-α–LT-β complex may also be a ligand for $TNFR_{60}$ (Fig. 1D). In contrast, $TNFR_{60}$:Fc (or $TNFR_{80}$:Fc) did not bind to II-23 cells 24 hours after activation (Fig. 1F), despite the presence of substantial antigen detectable with LT-α mAb (compare to Fig. 1B).

These findings raised the possibility that a receptor other than $TNFR_{80}$ and $TNFR_{60}$ may recognize the major form of cell surface LT and prompted us to test other members of the TNFR family (*3*) for binding to cell

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