Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 9302–9307, August 1997
Immunology

Abnormal development of secondary lymphoid tissues in lymphotoxin β-deficient mice

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Contributed by Klaus Rajewsky, May 22, 1997

ABSTRACT **The tumor necrosis factor (TNF) family cytokines lymphotoxin (LT) α and LTβ form heterotrimers that are expressed on the surface of activated lymphocytes and natural killer cells; LTα homotrimers can be secreted as well. Mice with a disrupted LTα gene lack lymph nodes (LN), Peyer's patches (PP), and follicular dendritic cell (FDC) networks and reveal profound defects of the splenic architecture. However, it is unclear which of these abnormalities is the result of the absence in LTα homotrimers or LTαβ heterotrimers. To distinguish between these two possibilities, a mouse strain deficient in LTβ was created employing Cre/loxP-mediated gene targeting. Mice deficient in LTβ reveal severe defects in organogenesis of the lymphoid system similar to those of LTα$^{-/-}$ mice, except that mesenteric and cervical LN are present in most LTβ-deficient mice. Both LTβ- and LTα-deficient mice show significant lymphocytosis in the circulation and peritoneal cavity and lymphocytic infiltrations in lungs and liver. After immunization, PNA-positive B cell clusters were detected in the splenic white pulp of LTβ-deficient mice, but FDC networks were severely underdeveloped. Collectively, these results indicate that LTα can signal independently from LTβ in the formation of PNA-positive foci in the spleen, and especially in the development of mesenteric and cervical LN.**

Lymphotoxin (LT) α, LTβ, and tumor necrosis factor (TNF) are structurally homologous cytokines grouped within the TNF ligand family (1). The genes for LTα, LTβ, and TNF are clustered within the major histocompatibility complex gene complex (2–4). TNF is produced by a variety of lymphoid and nonlymphoid cells as either membrane-bound or soluble homotrimers, both forms interacting with the two TNF receptors, TNFRp55 and TNFRp75 (1). LTα and LTβ are expressed by activated lymphocytes and natural killer cells (5), and both transcripts have been detected in the murine thymus and in the white pulp of the spleen (4). LTα lacks a transmembrane domain and is secreted as a homotrimer, yet it can be retained on the cell surface in heterotrimeric complexes with LTβ, a type II transmembrane protein (3). Although $LT\alpha_3$ shares receptors with TNF, the predominant surface $LT\alpha_1\beta_2$ heterotrimer was identified as a ligand for the LTβ receptor (LTβR) (6).

Given the ability of TNF and LTα to engage the same TNF receptors, it was difficult to assign distinct functions to these cytokines (7). Recent observations in gene-targeted mice deficient for TNFRp55 (8, 9), TNFRp75 (10), LTα (11, 12), TNF (13), or TNF/LTα (14, 15) have highlighted the different roles of TNF and LTα *in vivo*. TNF is a major mediator of septic shock and is involved in host defense against invading pathogens and in the generation of adaptive B cell immune responses (8, 9, 13). LTα is crucial for the development of lymph nodes (LN) and Peyer's patches (PP) and the organization of the white pulp of the spleen (11, 12). However, both TNF and LTα are indispensable for the formation of germinal centers and follicular dendritic cell (FDC) networks, which appears to be governed by TNFRp55 (13, 16).

Because both TNFRp55$^{-/-}$ and TNFRp75$^{-/-}$ mice develop LN and have normal spleen organization, it has been suggested that LTαβ heterotrimers control lymphoid organogenesis via the LTβR (17). In line with this assumption, administration of soluble LTβR fusion Fc chimeric protein into pregnant mice inhibited the genesis of PP and LN in developing embryos, with the exception of the mesenteric LN (18). In contrast, spleen organization was disrupted postnatally when soluble LTβR fusion protein was either expressed as a transgene or injected into mice (18, 19). In addition, development of PP also requires signaling via TNFRp55 (20).

To unambiguously dissect the function of membrane LTαβ from that of soluble LTα *in vivo*, we created a mouse strain deficient in LTβ and compared it with LTα$^{-/-}$ mice (11). Although some phenotypic alterations observed in LTβ-deficient mice were similar to those of LTα$^{-/-}$ mice, specific differences were observed. These results suggest that LTα has additional biological functions independent of LTβ.

MATERIALS AND METHODS

Targeting Vector. A murine genomic λ4 clone in EMBL3A (21) containing the genes for LTβ, TNFα, and LTα was used to construct the targeting vector. A 9-kB *Bam*HI-*Hin*dIII and a 4-kB *Hin*dIII-*Sal*I fragment (*Sal*I site from polylinker of EMBL3A) were subcloned into a modified pBSKS+ vector (Stratagene) resulting in pLTB1 and pLTA1, respectively. A cohesive end oligonucleotide duplex containing a synthetic loxP motif and a *Kpn*I site was inserted into the *Nde*I site of pLTB1, generating the pLTBlox1 vector. A *neo*-resistance gene cassette flanked by a single loxP motif was excised from pL2-neo (22) as *Xho*I fragment and cloned into the *Mlu*I site

Abbreviations: FDC, follicular dendritic cells; GC, germinal center; ES cells, embryonic stem cells; LN, lymph nodes; LTα, lymphotoxin α; LTβ, lymphotoxin β; LTβR, LTβ receptor; *neo*, *neomycin*-resistance gene cassette; PNA, peanut agglutinin; PP, Peyer's patches; TNF, tumor necrosis factor; TNFRp55, TNF receptor p55; TNFRp75, TNF receptor p75.

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of pLTBlox1 using a *Xho*I-*Mlu*I synthetic adapter. This vector, designated pLTlox2, was used as a positive control for establishing PCR conditions for embryonic stem (ES) cell screening. Construction of the targeting vector was continued by digesting pLTBlox2 with *Bam*HI and *Aat*II, blunting, and religating, resulting in pLTBlox2Δ. The long arm of homology was created by inserting a loxP-neo-loxP cassette derived from pL2-neo as *Sal*I-*Sph*I fragment into the modified *Kpn*I site of pLTA1, followed by removal of neo-loxP as a *Xho*I fragment and religation, yielding the pLTAlox1 vector. The fourth loxP site, together with a *Nde*I restriction site, was inserted into the *Hin*dIII site of pLTA1 as a cohesive end oligonucleotide duplex, resulting in pLTAlox2. The orientations of loxP motifs were verified by restriction analysis and sequencing. To assemble the final targeting vector, the genomic *Hin*dIII-*Sal*I fragment was recovered from pLTAlox2 and inserted into the corresponding sites of pLTBloxΔ, generating pTV2. Finally, the herpes simplex virus thymidine kinase gene cassette (TK) was excised from pTK as a *Sal*I-*Xho*I fragment and inserted into the *Sal*I site of pTV2. Both the targeting and the control constructs were verified by sequencing across all cloning sites using Sequenase (United States Biochemical) or the dsDNA Cycle Sequencing System (GIBCO/BRL).

Targeting of the TNF/LT Locus and Generation of LTβ-Deficient Mice. The targeting vector pTV2-TK was linearized with *Not*I and transfected into E14.1 ES cells by electroporation. ES cell transfection, culture, and selection was done as previously described (23). Four hundred G418- and gancyclovir-resistant colonies were picked and analyzed for homologous recombination by PCR using the primers 5′-TGA CCC TGT TGT TGG CAG TG-3′ and 5′-CCT GCG TGC AAT CCA TCT TG-3′. In 43 PCR-positive clones the correct recombination was verified by Southern blotting of genomic DNA. All 43 clones contained a correct single-copy insertion of the neo cassette, and 15 clones contained the second loxP motif without cointegration of the third or fourth loxP motif (data not shown). Because the exact location of the recombination breakpoint was not known, the coding sequences of the TNF gene of the targeted locus was specifically amplified from two clones by PCR and sequenced; no nucleotide alterations were noted. These two clones were selected for injection into C57BL/6 blastocysts and CD1 morula aggregation (24). Chimeric male mice were crossed to C57BL/6 females to obtain $LT\beta^{+/T}$ mice that were subsequently crossed to Cre-transgenic "deleter" mice (25), resulting in removal of the *neo*-cassette and exon 3 of the LTβ gene ($LT\beta^{+/\Delta}$). The progeny were genotyped by PCR for the $LT\beta^{\Delta}$ allele using the primers 5′-CGG GTC TCC GAC CTA GAG ATC-3′ and 5′-GAG GTG GGT GGA TTG GAA AGA G-3′. Correct Cre-mediated recombination was further confirmed by Southern blotting of *Bam*HI-digested genomic DNA using a *Pst*I-*Sph*I fragment as a flanking probe.

Animals. $LT\alpha^{-/-}$ mice (11) were obtained from The Jackson Laboratory. All mice were bred and housed in a conventional animal facility.

RNA Analysis. Total cellular RNA was extracted according to the protocol of Chomczynski and Sacchi (26). Twenty micrograms of RNA was loaded on a 1.5% formamide–agarose gel, blotted onto a nylon membrane (GeneScreen Plus, DuPont), and hybridized with PCR probes specific for LTβ, LTα, and TNFα cDNAs.

Immunohistochemistry. Immunohistochemistry was performed as described (20). The following rat anti-mouse monoclonal antibodies (Ab) were used: anti-B220, anti-CD11b, anti-Gr1, anti-MAdCAM-1 (PharMingen); MOMA-1 (Dianova, Hamburg, Germany); anti-FDC-M1 (4C11); anti-CD4 [American Type Culture Collection (ATCC), TIB-207]; and anti-CD8 (ATCC, TIB-105). Horseradish peroxidase-conjugated mouse anti-rat IgG was obtained from Jackson ImmunoResearch. For double staining, a biotinylated anti-CD3 mAb (PharMingen) and alkaline phosphatase-conjugated streptavidin (Sigma) were used. Horseradish peroxidase was developed with 3-aminoethylcabazole (Sigma), alkaline phosphatase was developed with the Vector Blue substrate kit (Vector Laboratories), and for single staining sections were counterstained with Mayer's hematoxilin, mounted with glycerol–gelatine, and photographed using a Leica DRMBE photomicroscope.

Double-immunofluorescent staining was performed on cryosections. B cell subsets were revealed using fluorescein isothiocyanate (FITC)-labeled anti-IgD and texas red (TR) labeled anti-IgM (Southern Biotechnology Associates). Germinal center cells were identified using biotinylated peanut agglutinin (PNA; Vector Laboratories; refs. 27 and 28), followed by avidin–FITC (Southern Biotechnology Associates) and TR-labeled IgM. The presence of FDC was assessed by using the rat anti-mouse FDC mAb, FDC-M2 (clone 209; ref. 29), followed by the F(ab′)2 mouse anti-rat IgG-specific antibody conjugated to FITC (Jackson ImmunoResearch) and TR-labeled anti-IgM. The sections were photographed using a Zeiss Axiophot.

Flow Cytometry. Erythrocyte-depleted single-cell suspensions from thymus, spleen, LN, peripheral blood, bone marrow, or peritoneal cavity lavage were treated with Fc-block (anti-CD16/CD32, PharMingen) and then incubated with antibodies by using standard procedures. The following antibody conjugates were used: biotin-145–2C11 (anti-CD3), R-phycoerythrin (PE)-RM4–5 (anti-CD4), biotin-53–7.3 (anti-CD5), biotin-M1/69 (anti-CD24), biotin-H1.2F3 (anti-CD69), PE-Mel-14 (anti-CD62L) (PharMingen); FITC-53–6.7 (anti-CD8), PE-RA3–6B2 (anti-B220), FITC-R33–24.12 (anti-IgM), FITC IgD antiserum (Nordic, Lausanne, Switzerland); and biotin-S7 (anti-CD43). Biotinylated Ab were detected with streptavidin–Cychrome (PharMingen). Thirty thousand events were analyzed within the lymphocyte gate as determined by forward and side scatter profiles on a FACScan (Becton Dickinson). Propidium iodide staining was used to exclude dead cells from the analysis.

Immunizations. Mice were immunized intraperitoneally with 2×10^8 sheep red blood cells (SRBCs) in 200 μl of PBS. Ten days later mice were sacrificed and spleens were prepared for immunohistochemistry.

Mouse immunoglobulin isotype-specific ELISAs were performed as described (30).

RESULTS

Inactivation of Mouse LTβ. The murine genes for LTα, LTβ, and TNF are tightly clustered within a 12-kb locus (4). A number of studies (31–33) have suggested that targeted mutations that retain a selectable marker cassette (e.g., *neo* gene under the control of the PGK I promoter) in a locus may yield unexpected phenotypes due to the altered expression of neighboring genes. Anticipating that the deficiency in LTβ will be phenotypically overlapping with the deficiency in LTα, it was critical that the targeted inactivation of the LTβ gene would not interfere with the expression of the LTα gene. A Cre/loxP-mediated gene-targeting approach (22) was employed to create a deletion of the third exon of the LTβ gene, which encodes most of the extracellular portion of the protein, 3′ untranslated region, and polyadenylylation signal (4). Even if a truncated protein was formed, it would lack most of the conserved domains believed to be important for trimerization and receptor binding (3, 34). A targeting vector was constructed to introduce loxP motifs into the mouse TNF/LT locus (see *Materials and Methods*). After homologous recombination, ES cell clones were identified with cointegration of loxP sites flanking the *neo*-cassette and the third exon of LTβ gene (Fig. 1*A*). Two independent ES cell clones were used to generate chimeric mice, which transmitted the targeted allele

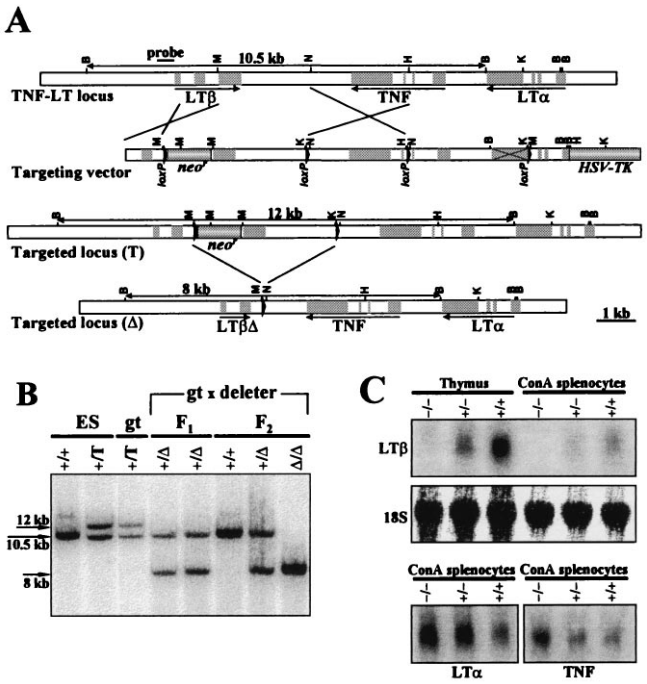

FIG. 1. Generation of LT$\beta^{\Delta/\Delta}$ mice. (*A*) Targeting strategy used for inactivation of the mouse LTβ gene. B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; N, *Nde*I. (*B*) Southern blot analysis of genomic DNA from targeted ES cells and from mouse tail biopsies. (*C*) Northern blot analysis of total RNA extracted from thymocytes or ConA-activated splenocytes derived from LT$\beta^{+/+}$, LT$\beta^{+/\Delta}$, and LT$\beta^{\Delta/\Delta}$ mice. 18S RNA was stained with methylene blue (loading control).

into the germline. To obtain the Cre/loxP-mediated deletion of the third exon of the LTβ gene together with the *neo*-cassette *in vivo*, LT$\beta^{+/T}$ mice were bred with transgenic "deleter" mice constitutively expressing Cre-recombinase in all cells early in development (25). Mice with the desired deletion (LT$\beta^{+/\Delta}$, Fig. 1*B*) were intercrossed. LT$\beta^{\Delta/\Delta}$ mice were born at the expected Mendelian frequency.

To ensure proper inactivation of the LTβ gene, Northern blot analysis was performed to check the expression of LTβ in thymocytes (4) and ConA-activated splenocytes (Fig. 1*C*). No RNA message for LTβ could be detected in cells from LT$\beta^{\Delta/\Delta}$ mice. The quantities of TNF and LTα transcripts were comparable in ConA-activated splenocytes derived from LT$\beta^{+/+}$, LT$\beta^{+/\Delta}$, and LT$\beta^{\Delta/\Delta}$ animals, providing evidence that the deletion of the major part of the LTβ gene did not grossly affect the regulation of the closely linked TNF and LTα genes (Fig. 1*C*).

Peripheral Lymphoid Organs in LT$\beta^{\Delta/\Delta}$ Mice. In view of the profound defects in peripheral lymphoid organs found in LTα mice (11), the development of lymphoid organs in LTβ-deficient mice was analyzed. Upon morphological inspection, no PP or brachial, axillary, inguinal, or popliteal LN could be detected. The absence of these LN was verified by histology. In marked contrast to LT$\alpha^{-/-}$ mice, most LT$\beta^{\Delta/\Delta}$ animals (about 75%) contained mesenteric LN. In addition, careful histological examination of LT$\beta^{\Delta/\Delta}$ animals revealed that many of them contained LN-like structures in the location of cervical LN (data not shown).

Immunohistochemical analysis of spleens of LT$\beta^{\Delta/\Delta}$ mice revealed marked alterations of the splenic architecture (Fig. 2*A*). Although T cells still appeared to accumulate around central arterioles, B cells did not form distinct follicles and were scattered in the white and red pulp of the spleen. Marginal zones were missing, as detected by the absence of MOMA-1$^+$ metallophilic macrophages (Fig. 2*A*) and MAdCAM-1 expression (data not shown). Immunofluorescent

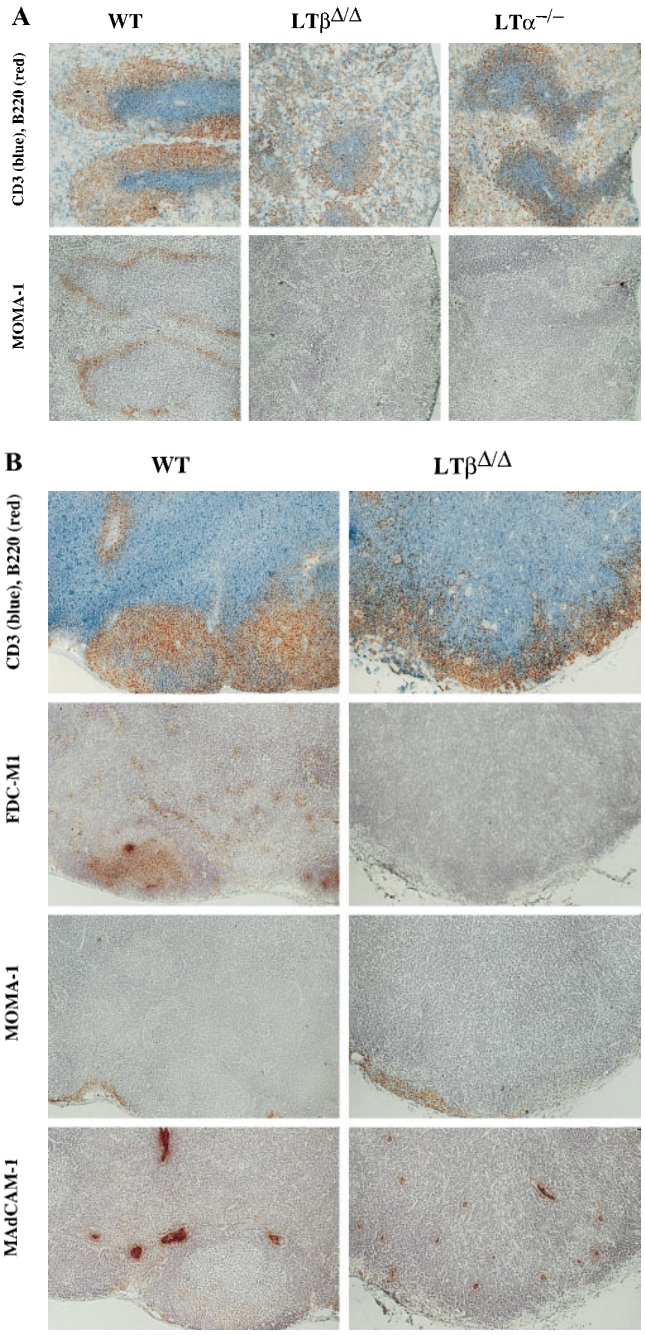

FIG. 2. Peripheral lymphoid organs in LT$\beta^{\Delta/\Delta}$ mice as compared with LT$\alpha^{-/-}$ mice (11). (*A*) Defective spleen organization in LT$\beta^{\Delta/\Delta}$ and LT$\alpha^{-/-}$ mice. Splenic cryosections of 6- to 8-week-old mice were processed to detect the distribution of T cells (CD3, blue) and B cells (B220, red) or metallophilic macrophages (MOMA-1). Original magnification, ×100. (*B*) Immunohistological analysis of mesenteric LN from LT$\beta^{\Delta/\Delta}$ mice. Serial sections were labeled with anti-CD3 (blue)/anti-B220 (red), anti-MOMA-1, anti-MAdCAM-1, or anti-FDC-M1 antibody.

microscopy of spleen sections employing antibodies against IgM and IgD revealed a difference in the staining patterns of IgM^{bright}D^{dull} cells and of IgM^{bright}D^{bright} cells in the white pulp. In control mice, a distinct area composed of IgM^{bright}-D^{dull} cells could be found in the marginal zone, whereas this type of organization could not be observed in LTα- or LTβ-deficient mice (data not shown). In general, segregation of lymphocytes in the white pulp into T and B cell areas appeared more conserved in LT$\beta^{\Delta/\Delta}$ than in LT$\alpha^{-/-}$ mice (Fig. 2*A*; data not shown). In contrast to the spleen, the

organization of the mesenteric LN in LT$\beta^{\Delta/\Delta}$ mice appeared generally unperturbed with a distinct subcapsular sinus containing MOMA-1$^+$ macrophages and normal segregation into T cell (paracortex) and B cell zones. However, B cell follicles were not well defined and lacked FDC networks, as judged by staining with FDC-M1 antibody (Fig. 2*B*). Interestingly, structures similar to high endothelial venules expressed MAdCAM-1 but appeared flattened compared with the high endothelial venules in control mice (Fig. 2*B*). Collectively, these data indicate that for the intact organization of the spleen and for the development of PP and some LN, LT$\alpha\beta$ heterotrimers are required. However, the formation of the mesenteric (and cervical, data not shown) LN can occur independently of LTβ.

Lymphocyte Populations in LT$\beta^{\Delta/\Delta}$ Mice. Lymphocyte populations in the thymus, bone marrow, spleen, mesenteric LN, blood, and peritoneal cavity were analyzed. Elevated numbers of lymphocytes were detected in the blood and in the peritoneal cavity, whereas the cellularity of the spleen (Table 1) and mesenteric LN (data not shown) was not altered in LT$\beta^{\Delta/\Delta}$ mice. Expression of L-selectin (Mel-14) was comparable on T and B cells from LT$\beta^{\Delta/\Delta}$, LT$\alpha^{-/-}$, and control mice (data not shown). Although LTβ and LTα were shown to be expressed in embryonic and adult murine thymus (4), no defects in thymocyte maturation were detected in LT$\beta^{\Delta/\Delta}$ or LT$\alpha^{-/-}$ mice by flow-cytometric analysis of thymocytes employing antibodies against CD3, CD4, CD5, CD8, CD24, and CD69 (data not shown). Also, maturation of B cells in the bone marrow appeared not to be affected in the absence of LTβ or LTα (data not shown).

Lymphocytic Infiltration of Parenchymal Organs. An extensive histopathological examination of organ systems of LT$\beta^{\Delta/\Delta}$ mice was performed. Strikingly, a marked accumulation of lymphocytes around perivascular areas was observed by HE staining of the lung and liver (data not shown). Immunohistochemical analysis revealed that cells in such aggregates were mainly B cells (B220$^+$) and CD4$^+$ T cells (Fig. 3). Very few CD8$^+$ T cells could be detected, and virtually no cells expressing Mac-1α or Gr-1 markers were observed (data not shown). A similar phenotype was also observed in LT$\alpha^{-/-}$ mice (Fig. 3 and ref. 12). Thus, in the absence of LTβ or LTα, predominantly CD4$^+$ T cells and B cells are abnormally recruited into tissues; however, it remains unclear why this occurs.

Lack of Germinal Centers in LT$\beta^{\Delta/\Delta}$ Mice. Despite the severe defects in secondary lymphoid organs, the levels of IgG in naive LT$\beta^{\Delta/\Delta}$, LT$\alpha^{-/-}$, or control mice were not significantly different, whereas IgM levels were evenly elevated in both mutant mice (data not shown). However, the proper framework of lymphoid tissues might be required for such an intricate immune reaction as germinal center formation. To investigate this in detail, mice were immunized with SRBC, a strong, thymus-dependent antigen. Germinal center and FDC development were assessed after 10 days by immunohistology. Consistent with previous studies (16), LT$\alpha^{-/-}$ mice did not form germinal centers, although rare aggregates of PNA-binding cells could be detected around central arterioles (Fig. 4). In marked contrast, distinct PNA-labeled cell clusters formed in the spleen (Fig. 4) and mesenteric LN (data not shown) of LT$\beta^{\Delta/\Delta}$ mice, although the size and the number of PNA$^+$ aggregates were reduced as compared with controls. Interestingly, some PNA-positive clusters were located in the periarteriolar lymphoid sheath around central arterioles, whereas other clusters were within B cell zones. Expression of the FDC-M1 antigen, a marker for mouse FDC, was observed neither in LT$\beta^{\Delta/\Delta}$ nor in LT$\alpha^{-/-}$ (data not shown). However, a few cells with dendritic morphology were clearly labeled with another anti-FDC monoclonal antibody, FDC-M2 (Fig. 4). In addition, immune-complex trapping assays on spleen sections from immunized LT$\beta^{\Delta/\Delta}$ mice revealed a greatly diminished, but conserved, capacity for antigen retention (data not shown). Thus, LTβ appears crucial for the full maturation of FDC networks and complete formation of germinal centers. How-

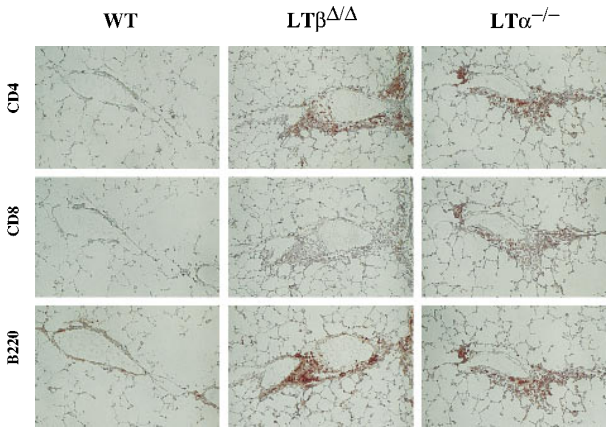

FIG. 3. Lymphocytic infiltrations in lungs of LT$\beta^{\Delta/\Delta}$ and LT$\alpha^{-/-}$ mice. Serial sections of frozen organs from 8-week-old mice were labeled with anti-CD4, anti-CD8, or anti-B220 antibody. Original magnification, ×100.

Table 1. Lymphocyte numbers in spleen, peritoneal cavity, and peripheral blood of LTβ- and LTα-deficient mice

	Experiment 1			Experiment 2		
	WT	LT$\beta\Delta/\Delta$	LT$\alpha-/-$	WT	LT$\beta\Delta/\Delta$	LT$\alpha-/-$
Spleen						
Cell number, × 10^6	130	100	150	130	140	130
Lymphocytes, %	45%	60%	61%	51%	53%	51%
B cells, × 10^6	18.7	25.8	43.0	24.5	35.6	19.9
Total T cells, × 10^6	13.6	17.7	24.1	10.2	19.0	20.6
CD4$^+$ T cells, × 10^6	8.2	12.6	17.4	6.6	13.0	12.6
CD8$^+$ T cells, × 10^6	5.4	5.1	6.7	3.6	6.0	8.0
Peritoneum						
Cell number, × 10^6	2.8	17	17	4.2	14	11
Lymphocytes	35%	71%	60%	40%	49%	49%
B cells, × 10^6	0.73	9.4	9.2	1.34	6.28	4.7
Total T cells, × 10^6	0.08	0.36	0.39	0.18	0.2	0.31
Blood						
White blood cells, × 10^5	4	24	50	8	45	18
B cells, × 10^5	0.7	9.4	25.5	3.6	23.9	4.9
T cells, × 10^5	2.8	13.2	22.0	3.6	16.2	12.4

WT, wild type.

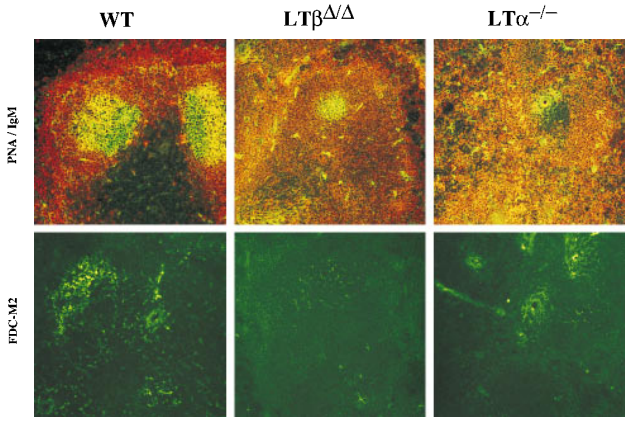

FIG. 4. Impaired FDC network development and germinal center formation in spleens of LT$\beta^{\Delta/\Delta}$ and LT$\alpha^{-/-}$ mice. Eight-week-old mice were immunized intraperitoneally with SRBC, and 10 days later spleens were removed. Immunofluorescence microscopy was performed on cryosections of spleens to detect formation of germinal centers (PNA-FITC IgM-Texas Red) and development of FDC networks (FDC-M2). Original magnification, ×100.

ever, the corresponding phenotype of LTβ-deficient mice appears less severe than that of LT$\alpha^{-/-}$ mice.

DISCUSSION

Since its discovery, the biological significance of cell surface LT (3, 5), a heterotrimeric complex of LTα and LTβ, has awaited clarification. Because the defects in LN development and spleen organization reported for LT$\alpha^{-/-}$ mice (11, 12) were not observed in mice deficient for TNFRp55 or TNFRp75 (8–10) (known receptors for the LTα homotrimer), it was suggested that LT$\alpha\beta$ interactions with the LTβ receptor regulate lymphoid organogenesis (17). Indeed, administration of a LTβR-Fc fusion protein into pregnant mice disrupted the formation of peripheral LN and Peyer's patches in offspring (18). Interestingly, genesis of mesenteric LNs was not affected by this treatment. However, it was not clear whether the anlage of mesenteric LN developed before the LTβR-Fc fusion protein could penetrate the placental barrier or whether the LT$\alpha\beta$ complex was not required for the development of the mesenteric LN.

We have used a gene-targeting approach to dissect the physiological role of LT$\alpha\beta$ complexes from that of LTα homotrimers. A mouse strain deficient for LTβ (LT$\beta^{\Delta/\Delta}$) was created and its phenotype was compared with that of LT$\alpha^{-/-}$ mice (11). We found that the majority of the phenotypic alterations was similar, including the lack of brachial, axillary, inguinal, popliteal LN, Peyer's patches, disrupted splenic architecture, elevated lymphocyte numbers in blood and peritoneum, and perivascular lymphocytic accumulations in lungs and liver. Thus, we conclude that these abnormalities are due to the loss of LT$\alpha\beta$ complexes. We also provide genetic evidence that development of mesenteric and cervical LN can take place in the absence of LT$\alpha\beta$ complexes.

An interesting concept of lymphoid neogenesis was developed by Kratz *et al.* (43) based on the studies of transgenic mice expressing LTα under the control of the rat insulin promoter. The authors showed that chronic inflammatory lesions that developed at the sites of the transgene expression displayed many features of organized lymphoid tissue, such as distinct T and B cell areas, presence of antigen presenting cells, secondary follicles, and high endothelial venules. Thus, formation of peripheral lymphoid organs (except the spleen) might be viewed as a developmentally fixed, local "inflammatory" reaction initiated mainly by LT$\alpha\beta$ heterotrimers.

The germinal center is a complex cellular microenvironment that supports the diversification of B cell receptors in activated B cells and the subsequent selection of B cells bearing receptors with increased affinity for the antigen (35, 36). The members of the TNF ligand family such as CD40L, TNF, and LTα were shown to play an important role in germinal center (GC) reaction (13, 16, 37, 38). A dual role for LTα in GC formation could be envisioned. First, in concert with LTβ, it could provide signals essential for the migration and/or differentiation of FDC in the primary follicles. Second, LTα is a well known growth factor for activated B cells (39), whose expression is induced in B cells after CD40 crosslinking (40). Thus, it might assist expansion of antigen-specific B cells, which takes place initially in periarteriolar lymphoid sheath and then in secondary follicles (41).

Finally, the development of mesenteric and cervical LN in LTβ-deficient mice described here suggests that the current model of ligand-receptor interactions between the three known ligands of the TNF/LT subfamily and the three known TNF/LT receptors may be incomplete. Because mice deficient in LTβR completely lack LN (K.P., unpublished data), thus revealing a phenotype comparable to LT$\alpha^{-/-}$ mice (11), LTα might form another heterotrimeric ligand, besides LT$\alpha\beta$, that is able to engage LTβR. Alternatively, development of mesenteric and cervical LN in LTβ-deficient mice could be explained as a result of low-affinity interaction between LTα homotrimers and LTβ receptors. The fact that this part of the phenotype of LTβ-deficient mice is not fully penetrant favors the latter suggestion. Analysis of mice deficient in both LTβ and TNF may help to clarify this question in the future.

LTβ-deficient mice have been independently generated as a collaboration between the laboratories of R. Flavell, N. Ruddle, and J. Browning (42), and the main features of the reported phenotype are similar to those described in this paper.

This work is a collaboration between the laboratories of K.P., S.N., and K.R.; it forms part of the Ph.D. thesis of M.A. We thank Drs. J. J. Oppenheim, R. Gilbert, and H. Wagner for critical reading of the manuscript; R. Kühn for E14.1 cells; F. Schwenk for providing transgenic "deleter" mice; and U. Huffstadt, K. Mink, E. Schaller, A. Futterer, S. Herren, and C. Hachenberg for technical assistance. We thank Drs. R. Flavell, J. Browning, and N. Ruddle for communicating results prior to publication. We thank the Frederick Biomedical Supercomputing Center at National Cancer Institute–Frederick Cancer Research and Development Center for allocation of computing time and staff support. This study was supported by grants from Sonderforschungsbereich 243, European Commission Biotechnology Program (CT94-2005), Deutsche Forschungsgemeinschaft (Pf259/2-3), Sonderforschungsbereich 391 project B3, the Klinische Forschergruppe "Postoperative Immunparalyse und Sepsis" project B2, the Russian State Program "Frontiers in Genetics," and the Russian Foundation for Basic Research (95-04-11854). The study also was supported by International Union Against Cancer fellowships to M.A. and R.T., and by the Russia Program of the Cancer Research Institute. S.N. and R.T. are International Research Scholars of the Howard Hughes Medical Institute.

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