

TNF and Lymphotoxin β Cooperate in the Maintenance of Secondary Lymphoid Tissue Microarchitecture But Not in the Development of Lymph Nodes¹

Dmitry V. Kuprash,^{2,3*†} Marat B. Alimzhanov,^{2*‡§} Alexei V. Tumanov,^{2*†} Arthur O. Anderson,[¶] Klaus Pfeffer,[†] and Sergei A. Nedospasov^{*†}

Inactivation of genes encoding members of TNF and TNF receptor families reveal their divergent roles in the formation and function of secondary lymphoid organs. Most lymphotoxin α (*lt α*)- and all lymphotoxin β receptor (*lt β r*)-deficient mice are completely devoid of lymph nodes (LNs); however, most lymphotoxin β (*lt β*)-deficient mice develop mesenteric LNs. *Tnf*- and *tnfrp55*-deficient mice develop a complete set of LNs, while *lt β /tnfrp55* double-deficient mice lack all LNs, demonstrating cooperation between LT β and TNFRp55 in LN development. Now we report that *lt β /tnf* double-deficient mice develop the same set of mucosal LNs as do *lt β* -deficient mice, suggesting that ligands other than TNF signal through TNFRp55 during LN development. These LNs retain distinct T and B cells areas; however, they lack follicular dendritic cell networks. Structures resembling germinal centers can be found in the LNs from immunized *lt β* -deficient mice but not in *lt β /tnf* double-deficient mice. Additionally, stromal components of the spleen and LNs appear to be more severely disturbed in *lt β /tnf* double-deficient mice as compared with *lt β* -deficient mice. We conclude that LT β and TNF cooperate in the establishment of the correct microarchitecture of lymphoid organs. *The Journal of Immunology*, 1999, 163: 6575–6580.

In recent years, a significant effort has been made to dissect lymphotoxin (LT)⁴ and TNF signaling using mice-deficient in TNF, LT, or their receptors as well as employing biochemical inactivation of LT/TNF signaling pathways (see Refs. 1 and 2; reviewed in Ref. 3). From in vitro studies, it is known that TNF₃ and LT α ₃ can bind to two TNF receptors p55 and p75, while

heterotrimeric LT α ₁/LT β ₂ is the main ligand for the LT β R (4). Specific contributions of LT/LT β R and TNF/TNFR signaling pathways to the development and the maintenance of microarchitecture of peripheral lymphoid organs are starting to emerge. Mice deficient in LT α (5, 6) lack proper T and B cell compartmentalization, marginal zone and FDC networks, and do not develop germinal centers (GC) in the spleen upon immunization. LT β -deficient mice have similar defects in spleen; however, T and B cell zones are not as mixed as in LT α -deficient mice (7, 8). TNF-deficient mice have even milder disturbances—marginal zone, albeit altered, is present in the spleen of these mice—but FDC networks are absent and GCs are not formed (9–11). Additionally, recent data by Alexopoulou et al. (12) suggested that TNF expression may be disturbed in the widely used *neo*-containing LT α -deficient mice and thus could have contributed to the severity of splenic white pulp disorganization.

TNF-deficient mice develop lymph nodes (LNs) normally, whereas LT α -deficient mice lack all LNs, except for abnormal lymphoid structures found in the mesenteric fat in 2–30% of mice (6, 13). LT β -deficient mice show an intermediate phenotype in this regard, they usually develop mesenteric LNs (MLNs), and, more rarely, sacral LNs (SLNs) and cervical LN. In these LNs, T and B cells segregate well and peanut agglutinin (PNA)-binding clusters are formed, but associated mature follicular dendritic cell (FDC) networks do not develop (7, 8, 14).

Defects in the spleen of LT β -deficient mice (7, 8) are more severe than in TNF^{-/-} mice (9). In contrast, several reports using biochemical rather than genetic inactivation of TNF (15, 16) suggested that TNF may play a distinct role in lymphoid organogenesis. Therefore, it was of considerable interest to determine whether a combined *neo*-free genetic excision of *lt β* and *tnf* genes may result in a more severe lymphoid deficiency as compared with single knockouts of the same genes.

In the present study, we generated and characterized a new mouse line with a combined TNF/LT deficiency, (LT β /TNF) ^{Δ/Δ}

*Laboratory of Molecular Immunology, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, and Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia; †Intramural Research Support Program, Science Applications International Corp.-Frederick and Laboratory of Molecular Immunoregulation, Division of Basic Sciences, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD, 21702; ‡Institute of Medical Microbiology, Immunology, and Hygiene, Technical University of Munich, Munich, Germany; §Institute for Genetics, University of Cologne, Cologne, Germany; and ¶Department of Clinical Pathology, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21702

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² D.V.K., M.B.A., and A.V.T. contributed equally to this work.

³ Address correspondence and reprint requests to Dr. Dmitry V. Kuprash, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov Street, 117984 Moscow, Russia. E-mail address: kupras@imb.ac.ru

⁴ Abbreviations used in this paper: LT, lymphotoxin; GC, germinal center; FDC, follicular dendritic cell; FRC, fibroblastic reticular cell; LN, lymph node; MLN, mesenteric LN; SLN, sacral LN; OPG, osteoprotegerin; PNA, peanut agglutinin; ES cell, embryonic stem cell; NIK, NF- κ B-inducing kinase.

double-deficient mice. We further dissected the contributions to the development and organization of peripheral lymphoid tissues of the two main signaling pathways involving LT and TNF. We show that under conditions where signaling both by LT $\alpha_1\beta_2$ via the LT β R and by TNF via the TNFRp55 are abrogated, two previously unrecognized effects become apparent: 1) redundant contributions of alternative ligands (i.e., LT α_3 and/or LIGHT) to the organogenesis of LNs, and 2) cooperative contributions of LT β R and TNFRp55 signaling to the microarchitecture of lymphoid organs.

Materials and Methods

Generation of LT β /TNF double-deficient mice

Construction of the targeting vector, embryonic stem (ES) cell transfection, and screening of homologous recombinants have been previously described (7). Of the 43 ES cell clones with a targeted insertion of the *neo* cassette, 15 clones revealed a coinjection of the second and the third loxP sites without coinjection of the fourth loxP motif of pTV2-TK. Two of these ES cell clones were used for injection into C57BL/6 blastocysts (17). Chimeric male mice were crossed to C57BL/6 mice to obtain germline transmission of the (LT β /TNF)^{+/-} mutation. (LT β /TNF)^{+/-} mice were crossed to Cre-transgenic "deleter" mice with ubiquitous expression of Cre recombinase (18). As a result, the *neo*-cassette and a region of the TNF/LT locus containing exon 3 of the *ltb* gene and exons 3 and 4 of the *tnf* gene were deleted in the mutant allele. The progeny was genotyped by PCR using primers gtype1, 5'-CGG GTC TCC GAC CTA GAG ATC and gtype5a, 5'-CAG ACC CTC ACA CTC AGT AAG. Correct excision of the portion of the TNF/LT locus was further confirmed by Southern analysis using BamHI digestion of genomic DNA and the *SphI-PstI* portion of the LT β promoter as a probe. The Cre-transgene was crossed out during further backcrossing to C57BL/6 mice. Heterozygous mice were bred to obtain (LT β /TNF)^{ΔΔ} double-deficient mice, which were then embryo derived and maintained under specific pathogen-free conditions.

Animals

All mice were generated on a mixed C57/BL6/129cv background and then backcrossed to C57/BL6. LT α ^{-/-} mice (5) were purchased from The Jackson Laboratory (Bar Harbor, ME). LT β -deficient mice (LT β ^{ΔΔ} mice) have been described previously (7). All mice were maintained under specific pathogen-free conditions and used for experiments between 8 and 12 wk of age. Animal care was provided in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health Publication No. 86-23, 1985).

RNA analysis

Total cellular RNA was extracted with Trizol reagent and used as described by the manufacturer in RNAase protection assays with the mCK-3 set of probes, including *lt α* , *ltb*, and *tnf* genes (PharMingen, San Diego, CA).

Immunizations

Where indicated, mice received a single i.p. injection of 50 μ g of alum-precipitated nitrophenol-haptenated chicken γ -globulin or 10⁸ SRBC in PBS and were analyzed 8 days after immunization.

Immunohistochemistry

Immunohistochemical analysis was performed as described (2, 7). Hamster Abs to CD3 α and rat Abs to B220, IgD, IgM, CR1, and mucosal addressin cell adhesion molecule-1 were obtained from PharMingen. Biotinylated PNA and the alkaline phosphatase staining kit were purchased from Vector Laboratories (Burlingame, CA). ER-TR7 mAb was purchased from Biogenesis (Poole, U.K.). MOMA-1 mAb was purchased from Research Diagnostics (Flanders, NJ). FDC-M1 Abs were generously provided by Dr. M. Kosco-Vilbois (Serono Pharmaceutical Research Institute, Geneva, Switzerland). Peroxidase-conjugated mouse anti-rat IgG Abs (F(ab')₂) were obtained from Jackson ImmunoResearch (West Grove, PA). Alkaline phosphatase-conjugated streptavidin and other reagents were obtained from Sigma (St. Louis).

Flow cytometry

Flow cytometric analysis was performed on single-cell suspensions as described (2). Biotin-, FITC-, or PE-conjugated rat Abs to the following mouse surface markers were used: Thyl.2 (clone 30H12), CD4 (clone

RM4-5), and CD8 (clone 53-6.7) to detect T cells; B220 (clone RA3-6B2) for B cells; and Gr1 (clone RB6-8C5) and Mac1 α (clone M1/70) for granulocytes and macrophages. All primary Abs were obtained from PharMingen.

Results and Discussion

Generation of LT β /TNF double-deficient mice

To address the possible redundancy of TNF/LT ligands in the development of peripheral lymphoid organs we have generated a mouse strain deficient in both LT β and TNF. Because the *tnf* and *lt* genes are closely linked (19–21) it would be virtually impossible to generate such mice by crossing single TNF and LT β knockout mice. Therefore, (LT β /TNF)^{ΔΔ} mice were generated using Cre-loxP technology, similarly to the previously described LT β ^{ΔΔ} mice (7). In particular, we generated ES cells with a partial homologous recombination of the targeting vector pTV2-TK (7) and used them for injection into C57BL/6 blastocysts. After germline transmission, Cre-mediated recombination in vivo using "deleter" mice (18) was employed to remove the selectable marker (*neo* gene) and to create a 5.3-kb deletion (Fig. 1A). This deletion (confirmed by Southern analysis; Fig. 1B) included the third exon of the *ltb* gene (encoding most of the extracellular portion of this cytokine) and the third and the fourth exons of the *tnf* gene (encoding the entire secreted portion of the TNF protein) and did not allow the expression of functional LT β and TNF mRNAs.

Mice with the double LT β /TNF deficiency were born at the expected Mendelian ratios, appeared healthy, and were fertile. Thymi had normal weight and appearance, and the major cell populations in thymus and bone marrow were normal as measured by FACS analysis (data not shown). Lymphoid infiltrates were found around blood vessels in lung and liver of (LT β /TNF)^{ΔΔ} mice (data not shown), similarly to the infiltrates described for LT β ^{ΔΔ} mice (7), LT α ^{-/-} mice (6), and LT β R^{-/-} mice (2). Transcription of the *lt α* gene was not disturbed by the deletion, as confirmed by RNase protection assay performed with total RNA isolated from Con A-activated splenocytes (Fig. 1C).

TNF and LT β do not cooperate in LN organogenesis

Peripheral lymphoid organs of (LT β /TNF)^{ΔΔ} mice were examined. No visible Peyer's patches were found and no inguinal, brachial, axillary, iliac, or popliteal LN could be detected by histopathological examination (data not shown). However, MLNs and sometimes SLNs could be readily detected (in 15 of 17 and 6 of 17 mice, respectively). Thus, lymphoid organs in (LT β /TNF)^{ΔΔ} mice develop similarly to the previously described LT β ^{ΔΔ} mice (MLNs, 15 of 23; SLNs, 6 of 23). The anatomical shape of MLNs was unusual in both single *ltb*- and double *ltb/tnf*-deficient animals. In most cases, only one large, spherical MLN was found in mutant mice at a point where the root of the mesentery merges with the parietal peritoneal mesothelium, as opposed to a chain of three elongated LN segments normally present along the mesenteric artery in wild-type mice. The specific anatomical location of the MLN found in LT β ^{ΔΔ} and (LT β /TNF)^{ΔΔ} mice suggests that some of the segments of the MLN draining the lymph from the gut (small bowel, cecum, and sigmoid colon) may be missing in these animals.

MLNs from (LT β /TNF)^{ΔΔ} mice were examined by immunohistology and compared with MLNs from wild-type mice and from LT β ^{ΔΔ} mice (Fig. 2). As expected, FDC networks were absent in all mutant mice as assessed by staining for CR1 (Fig. 2) or FDC-M1 (data not shown).

Additional abnormalities of the MLN microarchitecture were observed in the (LT β /TNF)^{ΔΔ} mice as compared with LT β ^{ΔΔ}

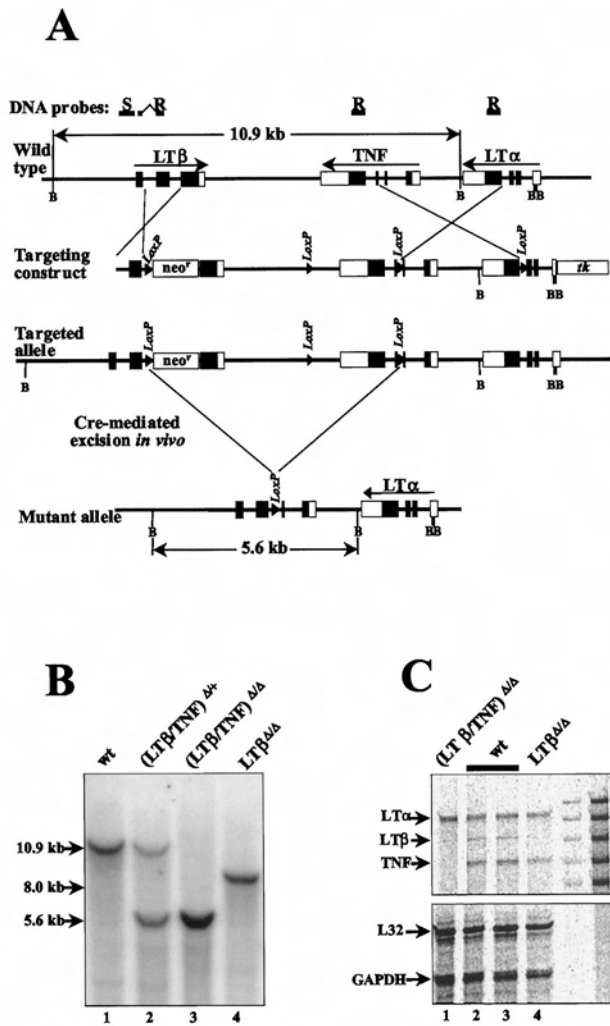


FIGURE 1. Generation of $(LT\beta/TNF)^{\Delta\Delta}$ mice. *A*, Map of the targeting construct, wild-type, targeted, and mutant $LT\beta/TNF$ allele. Location of DNA probes used in Southern (“S”) and RNase protection (“R”) experiments is shown above the map of the wild-type locus. *B*, Southern analysis. Genomic DNA from the wild-type or indicated mutant mice was digested with *Bam*HI, and the blot was hybridized with a fragment of the murine $LT\beta$ promoter (probe “S” in *A*). *C*, RNase protection assay. Total splenocytes were prepared from the mice with the indicated genotype. Cells were cultured for 12 h and stimulated with 5 μ g/ml Con A for an additional 12 h. Approximately 10 μ g of total RNA prepared with Trizol reagent was used per lane. The location of the probes for TNF, $LT\alpha$, and $LT\beta$ transcripts used in this assay is shown in *A* (probes “R”). Note that our gene inactivation strategy did not remove the entire $lt\beta$ gene, and it was possible to detect an aberrant $LT\beta$ transcript with this particular $lt\beta$ probe.

mice. Both types of mice retained a distinct segregation of lymphocytes into a superficial cortical B cell zone and a deep cortical T cell zone. Furthermore, $LT\beta^{\Delta\Delta}$ mice were able to form structures in the superficial cortex that resembled GCs in MLN upon immunization (Fig. 2), in agreement with the recent report by Koni and Flavell (14). Because $CR1^+$ FDC networks appeared to be absent in $LT\beta^{\Delta\Delta}$ mice, it is not possible to state with certainty if these GC-like structures were segregated into light zone or dark zone compartments. However, small IgD^+/IgM^+ lymphocyte coronas and IgM^+/PNA^+ GCs were found both in unstimulated and in Ag-stimulated mice (data not shown).

In contrast to $LT\beta^{\Delta\Delta}$ mice, PNA^+ B cells found in the LNs from $(LT\beta/TNF)^{\Delta\Delta}$ mice were scattered and did not form foci

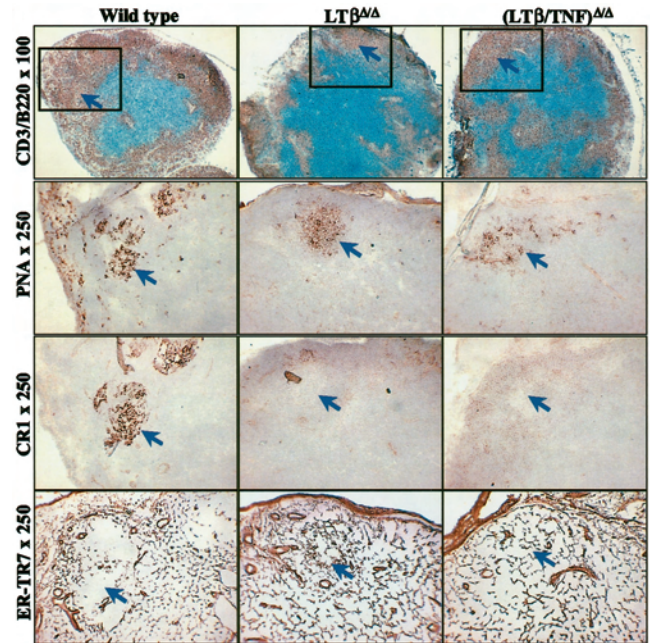


FIGURE 2. Immunohistology of LNs. Serial frozen sections of MLNs from SRBC-immunized mice were labeled with the indicated Abs. The images are grouped in columns by genotype and in rows by label. Original magnification of each row is indicated on the left. In the top row, CD3 is blue and B220 is red. Arrows indicate positions of PNA^+ cell clusters in B cell areas.

resembling GCs. There was segregation of $B220^+$ B cells to the superficial cortex and of $CD3^+$ T cells to the deep cortex of MLN, but in areas where one would expect to find B cell follicles, only scattered individual PNA^+ B cells were found (Fig. 2).

Examination of the underlying fibroblastic reticular cell (FRC) architecture with ER-TR7 Ab (22) provided useful correlates. In wild-type mice, there was a delicate FRC network in the deep cortex (paracortex) that formed the narrow perivenular channel, FRC corridors, and cortical columns that are characteristic of this compartment (23). In the superficial cortex, this delicate pattern undergoes an abrupt transition where there are interfaces with B cell follicles (Fig. 2, bottom row). The corona usually retains the delicate pattern of the paracortex but GCs are separated from the corona by an FRC “barrier” and the GC areas contain broad zones that are relatively free of fibroblastic reticulum. This may presumably be to accommodate the FDC network. Regardless of the reason, the FRC pattern usually reflects the presence of structures like GCs.

In the $LT\beta^{\Delta\Delta}$ mice, the reticulum of the deep cortex was coarser than in the wild-type mice, with thicker fibers and wider spaced corridors. In these mice, the transition of FRC at GC-like structures was less distinct than that seen in the wild-type mice, but some alteration in the reticulum pattern in the areas of PNA^+ B cell foci was evident, with a ring of more dense stromal components surrounding PNA^+ area (Fig. 2, bottom row). In contrast, in $(LT\beta/TNF)^{\Delta\Delta}$ mice the coarse pattern of the fibroblastic reticulum was uniform across the T-dependent and B-dependent cortex and no GC-like architectural transitions were seen. Apparently, the loosely scattered PNA^+ B cells were of insufficient aggregate size to have an impact on the underlying reticulum, so the cortical pattern extended to the subcapsular sinus.

We concluded that PNA^+ B cell clusters resembling GCs can be formed in the absence of FDC network (as assessed by CR1 and

Table I. Secondary lymphoid organs in TNF/LT knockout mice^a

Genotype	Splenic Architecture			Peyer's Patch	LN	References
	Marginal zone	T/B cell areas	GC/FDC			
<i>ltα</i> ^{-/-}	Absent	Mixed	No	Absent	None	5, 6
<i>ltβr</i>	Absent	Mixed	No	Absent	None	2
<i>ltβ</i> ^{-/-}	Absent	Segregated	No	Absent	Mucosal	7, 8
<i>ltβ</i> ^{-/-} <i>tnfrp55</i> ^{-/-}	NR ^b	NR	NR	Absent	None	24
<i>ltβ</i> ^{-/-} <i>tnf</i> ^{-/-}	Absent	Variable	No	Absent	Mucosal	This work
<i>tnfrp55</i> ^{-/-}	Abnormal	Segregated	No	Disturbed	All	43–45
<i>tnf</i> ^{-/-}	Abnormal	Segregated	No	Small	All	9, 10

^a The features of the secondary lymphoid organs of (LT β /TNF) $\Delta\Delta$ mice are highlighted by comparing them to several selected knockout mice with deficiencies in TNF/LT and their receptors.

^b NR, Not reported.

FDC-M1 staining) in LT β $\Delta\Delta$ but not in (LT β /TNF) $\Delta\Delta$ mice, suggesting independent contribution of TNF into underlying signaling mechanism. We also concluded that stromal components of the MLN were more severely disturbed in the double knockout.

In an attempt to characterize cervical LNs in (LT β /TNF) $\Delta\Delta$ mice, we performed immunohistological examination of LN-like structures found in the normal location of cervical LNs (data not shown). However, we were unable to identify any true cervical LNs either in (LT β /TNF) $\Delta\Delta$ mice ($n = 5$) or in LT β $\Delta\Delta$ mice ($n = 7$). This result, which is somewhat contradictory to the published observations (7, 8), may be due to the fact that the mice used in this study have been rederived by embryo transfer and maintained under specific pathogen-free conditions thereafter or due to a low incidence of cervical LNs in this mouse model.

The double *ltb* and *tnfrp55* knockout mice (24) lack all LNs, including MLN and SLN (see Table I). Because inactivation of the TNFRp55 gene disrupts signaling by both TNF and LT α_3 , the comparison with the (LT β /TNF) $\Delta\Delta$ model suggests that it is binding of LT α_3 , and not of TNF, to TNFRp55 that is important for cooperation with LT β R signaling for the development of the mucosal subset of LNs.

Additionally, Rennert et al. (1) used injections of LT β R-Ig and TNFRp55-Ig or LT β R-Ig and anti-TNF mAb into pregnant mice to inactivate simultaneously both signaling pathways. Results with the first combination of antagonists are in agreement with genetic data (24).

However, the absence of LNs from wild-type mice simultaneously injected in utero with LT β R-Ig and anti-TNF mAb (1) is in apparent contradiction with our results. Several explanations can be offered. First, if effects of TNF are mediated by cell-surface-bound ligands, then TNF-bearing cells may be lysed in the Ab-injection experiment by a complement-mediated mechanism. Second, LN genesis may involve an additional signaling through LT β R by LIGHT (25) or by another as yet unidentified ligand, and such signaling would be disrupted in the model of Rennert et al. but not in (LT β /TNF) $\Delta\Delta$ mice. This hypothesis is further supported by the report on the mice with genetic inactivation of LT β R (2), which do not develop any LNs.

Collectively, our data support the cooperation of TNFRp55 and LT β R signaling cascades in organogenesis of LNs and suggest a distinct role for LT α_3 -TNFRp55 signaling. Together with observations by Koni and Flavell (24), this represents a novel biological function of LT α_3 detected in vivo in a nontransgenic model. Previously evidence for distinct function of LT α_3 -TNFRp55 interaction in lymphoid organogenesis and inflammation was provided by a series of elegant studies using LT α transgene under the control of the rat insulin promoter (26–29). The hypothesis concerning the role of an additional LT α -specific receptor in LN development

(30) is not supported by the phenotype of *tnfrp55*^{-/-}*ltb*^{-/-} (24) or (LT β /TNF) $\Delta\Delta$ mice (this report).

The development of LNs is a complex process involving several cell lineages and multiple signaling pathways. Nevertheless, the role of LT-LT β R has been reinforced by the findings on *aly/aly* mice (31) in which case the defect is caused by mutation in carboxyl-terminal end of NF- κ B-inducing kinase (NIK) (32). This result also places NIK (33) in the signaling cascade downstream of LT β R (and probably of some other receptors of the TNFR superfamily) and not of TNFRp55 (32). Additionally, *opgl*^{-/-} mice (34) also completely lack LNs. Osteoprotegerin (OPG) ligand (OPGL) (also known as TNF-related activation-induced cytokine (TRANCE), receptor activator of NF- κ B ligand (RANKL), or osteoclast differentiation factor (ODF); Refs. 35–37) is a ligand for OPG and RANK that is also a member of the TNFR superfamily. It is possible that the OPGL/(OPG/RANK) signaling pathway shares some downstream molecules with LT β R signaling pathways (see Ref. 35). Whether NIK is involved in this signaling remains to be demonstrated. Alternatively, OPG/RANK signaling may be required at a different step in LN development.

TNF and LT β cooperate in the maintenance of splenic microarchitecture

Spleens of (LT β /TNF) $\Delta\Delta$ mice were studied by immunohistochemistry after i.p. immunization with T cell-dependent Ags SRBC (Fig. 3) or nitrophenol-haptenated chicken γ -globulin (data not shown). Wild-type littermate controls, LT β $\Delta\Delta$ (7), and LT α ^{-/-} (5) mice were examined in parallel (Table I). As a common feature, FDC networks could not be detected in any of the mutant mice using staining for CR1 (Fig. 3) or FDC-M1 (data not shown), and there were no clustered PNA-positive cells in B cell areas, although some aberrant PNA staining with a variable pattern could be detected around central arterioles and blood vessels (Fig. 3). In agreement with the published data, the staining of the marginal zone of the spleen by mucosal addressin cell adhesion molecule-1 and MOMA-1 markers was absent in (LT β /TNF) $\Delta\Delta$, LT β $\Delta\Delta$, and LT α ^{-/-} mice (data not shown).

Importantly, the overall splenic structure was more severely disturbed in the double-deficient mice, as compared with single LT β knockouts (Fig. 3 and Table I). Spleens of LT β $\Delta\Delta$ mice still retained relatively distinct B cell zones, while in the double-deficient mice B cell areas were smaller and much less defined (Fig. 3), even though the numbers of B and T cells in the spleen of the double (LT β /TNF) $\Delta\Delta$ mice were not changed as assessed by flow cytometry analysis (data not shown). The relative numbers of T and B cells in spleen of (LT β /TNF) $\Delta\Delta$ mice (15–20% and 30–35%, respectively) as well as the absolute numbers of lymphocytes in spleen (0.8 – 1.5×10^8) and in thymus (1.3 – 1.7×10^8) were not

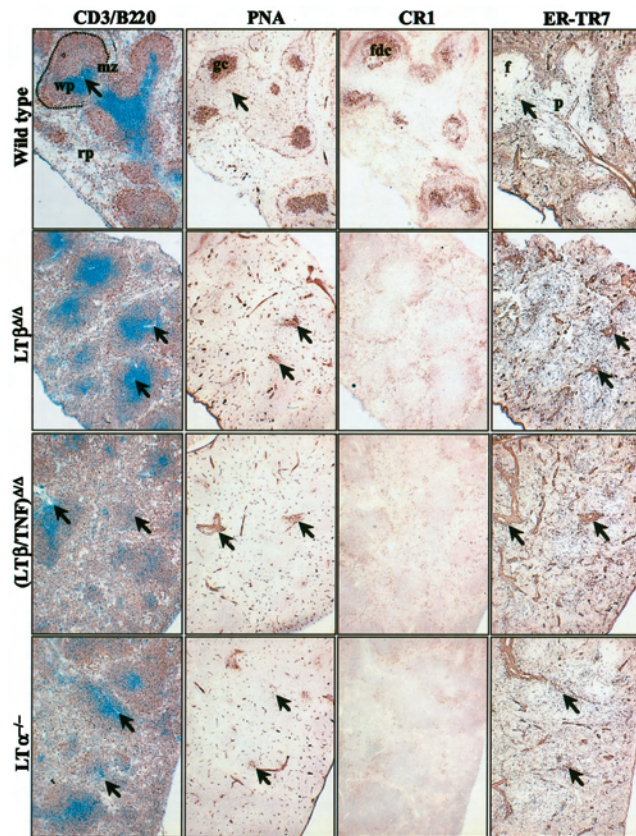


FIGURE 3. Immunohistology of spleen. Serial frozen sections of spleens from SRBC-immunized mice were stained with the indicated Abs. The images are grouped in rows by genotype and in columns by label. In the left column, CD3 is blue and B220 is red. Original magnification, $\times 100$. wp, white pulp; rp, red pulp; mz, marginal zone; gc, germinal center; fdc, follicular dendritic cell network; p, periarteriolar lymphoid sheath; f, B cell follicle. Arrows indicate positions of central arterioles.

significantly different from the wild-type mice, while the white cell counts in blood and peritoneum were two to three times higher in $(LT\beta/TNF)^{\Delta/\Delta}$ mice as compared with the wild-type mice, the phenomenon previously described for $LT\alpha^{-/-}$ (5, 6) and $LT\beta^{\Delta/\Delta}$ mice (7, 8).

The profound defect of the splenic T/B cell segregation was further characterized using labels specific for the stromal components of the spleen. Labeling with ER-TR7 Ab, which detects reticular fibroblasts and blood vessel walls (22), revealed that stromal components of the spleen that support spatial organization of the lymphoid tissues (38) were more disturbed in $(LT\beta/TNF)^{\Delta/\Delta}$ mice than in $LT\beta^{\Delta/\Delta}$ mice (Fig. 3). ER-TR7-positive stromal elements in the spleen of $LT\beta^{\Delta/\Delta}$ mice, even though markedly disorganized in comparison with the wild type, still formed a distinct ellipsoid-like structure confining the white pulp area. In contrast, ER-TR7 labeling of the spleen of $(LT\beta/TNF)^{\Delta/\Delta}$ mice did not reveal a clear boundary between the red and white pulp (Fig. 3). Routine chemical stains for elastic or collagen fibers (Orcein and Masson's TriChrome) (39) revealed an increased density of these connective tissue elements around the central arterioles in the spleen of $(LT\beta/TNF)^{\Delta/\Delta}$ mice as compared with $LT\beta^{\Delta/\Delta}$ and wild-type mice (Fig. 4). This accumulation of the connective tissue around central arterioles was not due to insufficient number of T cells (see above) and may be responsible for the deficient compartmentalization by affecting lymphocyte migration or by providing dislocated chemotactic or adhesion signals (40).

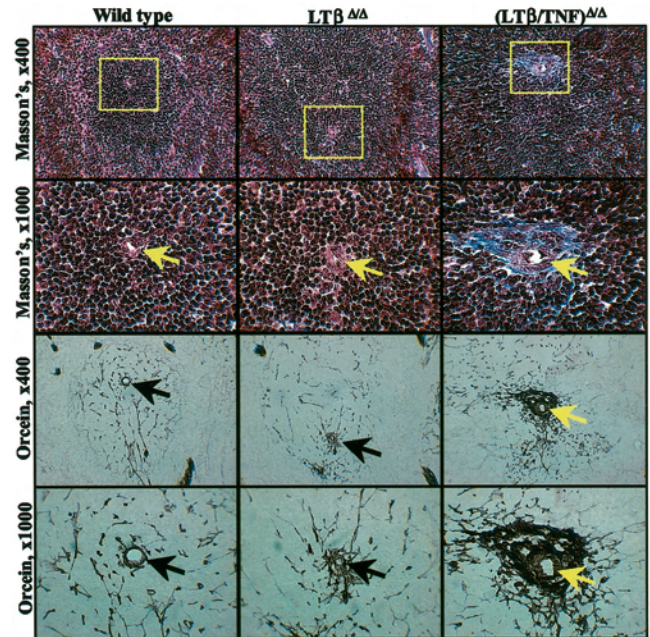


FIGURE 4. Histochemistry of spleen. Serial sections of paraffin-embedded spleens from SRBC-immunized mice were stained either with Masson's TriChrome (top) or with Orcein stain (bottom). Masson's TriChrome stains collagen fibers with blue color; Orcein stains reticular and elastic fibers. Original magnification of each row is indicated on the left side. Arrows indicate central arterioles.

Expression levels of certain chemokines also play an important role in forming functional compartments of the spleen. Mice deficient in one of the chemokine receptors, BLR-1/CXCR5 (41), have defects in their peripheral lymphoid tissues that are reminiscent, although not as severe, as those reported for $LT\alpha$ - or $LT\beta$ -deficient mice. A substantial decrease with regard to expression levels of the B lymphocyte chemoattractant and of the secondary lymphoid tissue chemokine reported in association with $LT\alpha$ deficiency (42) is also observed in both $(LT\beta)^{\Delta/\Delta}$ and $(LT\beta/TNF)^{\Delta/\Delta}$ mice (without substantial difference between these two models, data not shown). In the future, it will be interesting to determine whether any of these chemokine genes may be under direct control of $LT\beta R$ or $TNFR55$ signaling.

In summary, our genetic data demonstrate that there is an independent contribution of TNF to the LT-dependent maintenance of the microarchitecture of the spleen and LN. Our data also suggest a distinct role of $LT\alpha_3$ - $TNFRp55$ interaction in the development of mucosal LNs.

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References

1. Rennett, P. D., D. James, F. Mackay, J. L. Browning, and P. S. Hochman. 1998. Lymph node genesis is induced by signaling through the lymphotoxin β receptor. *Immunity* 9:71.

2. Futterer, A., K. Mink, A. Luz, M. H. Kosco-Vilbois, and K. Pfeffer. 1998. The lymphotoxin β receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* 9:59.
3. Chaplin, D. D., and Y. Fu. 1998. Cytokine regulation of secondary lymphoid organ development. *Curr. Opin. Immunol.* 10:289.
4. Ware, C. F., T. L. VanArsdale, P. D. Crowe, and J. L. Browning. 1995. The ligands and receptors of the lymphotoxin system. *Curr. Top. Microbiol. Immunol.* 198:175.
5. De Togni, P., J. Goellner, N. H. Ruddle, P. R. Streeter, A. Fick, S. Mariathasan, S. C. Smith, R. Carlson, L. P. Shornick, J. Strauss-Schoenberger, J. H. Russell, R. Karr, and D. D. Chaplin. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 264:703.
6. Banks, T. A., B. T. Rouse, M. K. Kerley, P. J. Blair, V. L. Godfrey, N. A. Kuklin, D. M. Bouley, J. Thomas, S. Kanangat, and M. L. Mucenski. 1995. Lymphotoxin- α -deficient mice: effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155:1685.
7. Alimzhanov, M. B., D. V. Kuprash, M. H. Kosco-Vilbois, A. Luz, R. L. Turetskaya, A. Tarakhovskiy, K. Rajewsky, S. A. Nedospasov, and K. Pfeffer. 1997. Abnormal development of secondary lymphoid tissues in lymphotoxin β -deficient mice. *Proc. Natl. Acad. Sci. USA* 94:9302.
8. Koni, P. A., R. Sacca, P. Lawton, J. L. Browning, N. H. Ruddle, and R. A. Flavell. 1997. Distinct roles in lymphoid organogenesis for lymphotoxins α and β revealed in lymphotoxin β -deficient mice. *Immunity* 6:491.
9. Pasparakis, M., L. Alexopoulou, V. Episkopou, and G. Kollias. 1996. Immune and inflammatory responses in TNF α -deficient mice: a critical requirement for TNF α in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J. Exp. Med.* 184:1397.
10. Marino, M. W., A. Dunn, D. Grail, M. Inglese, Y. Noguchi, E. Richards, A. Jungbluth, H. Wada, M. Moore, B. Williamson, S. Basu, and L. J. Old. 1997. Characterization of tumor necrosis factor-deficient mice. *Proc. Natl. Acad. Sci. USA* 94:8093.
11. Sean Riminton, D., H. Korner, D. H. Strickland, F. A. Lemckert, J. D. Pollard, and J. D. Sedgwick. 1998. Challenging cytokine redundancy: inflammatory cell movement and clinical course of experimental autoimmune encephalomyelitis are normal in lymphotoxin-deficient, but not tumor necrosis factor-deficient, mice. *J. Exp. Med.* 187:1517.
12. Alexopoulou, L., M. Pasparakis, and G. Kollias. 1998. Complementation of lymphotoxin α knockout mice with tumor necrosis factor-expressing transgenes rectifies defective splenic structure and function. *J. Exp. Med.* 188:745.
13. Fu, Y. X., G. Huang, M. Matsumoto, H. Molina, and D. D. Chaplin. 1997. Independent signals regulate development of primary and secondary follicle structure in spleen and mesenteric lymph node. *Proc. Natl. Acad. Sci. USA* 94:5739.
14. Koni, P. A., and R. A. Flavell. 1999. Lymph node germinal centers form in the absence of follicular dendritic cell networks. *J. Exp. Med.* 189:855.
15. Ettinger, R., R. Mebius, J. L. Browning, S. A. Michie, S. van Tuijl, G. Kraal, W. van Ewijk, and H. O. McDevitt. 1998. Effects of tumor necrosis factor and lymphotoxin on peripheral lymphoid tissue development. *Int. Immunol.* 10:727.
16. Mackay, F., G. R. Majeau, P. Lawton, P. S. Hochman, and J. L. Browning. 1997. Lymphotoxin but not tumor necrosis factor functions to maintain splenic architecture and humoral responsiveness in adult mice. *Eur. J. Immunol.* 27:2033.
17. Joyner, A. L. 1993. *Gene Targeting: A Practical Approach*. IRL PRESS at Oxford University Press, New York.
18. Schwenk, F., U. Baron, and K. Rajewsky. 1995. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res.* 23:5080.
19. Nedospasov, S. A., B. Hirt, A. N. Shakhov, V. N. Dobrynin, E. Kawashima, R. S. Accolla, and C. V. Jongeneel. 1986. The genes for tumor necrosis factor (TNF- α) and lymphotoxin (TNF- β) are tandemly arranged on chromosome 17 of the mouse. *Nucleic Acids Res.* 14:7713.
20. Browning, J. L., A. Ngam-ek, P. Lawton, J. DeMarinis, R. Tizard, E. P. Chow, C. Hession, B. O'Brine-Greco, S. F. Foley, and C. F. Ware. 1993. Lymphotoxin β , a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell* 72:847.
21. Pokholok, D. K., I. G. Maroulakou, D. V. Kuprash, M. B. Alimzhanov, S. V. Kozlov, T. I. Novobrantseva, R. L. Turetskaya, J. E. Green, and S. A. Nedospasov. 1995. Cloning and expression analysis of the murine lymphotoxin β gene. *Proc. Natl. Acad. Sci. USA* 92:674.
22. Van Vliet, E., M. Melis, and W. van Ewijk. 1984. Monoclonal antibodies to stromal cell types of the mouse thymus. *Eur. J. Immunol.* 14:524.
23. Gretz, J. E., A. O. Anderson, and S. Shaw. 1997. Cords, channels, corridors and conduits: critical architectural elements facilitating cell interactions in the lymph node cortex. *Immunol. Rev.* 156:11.
24. Koni, P. A., and R. A. Flavell. 1998. A role for tumor necrosis factor receptor type 1 in gut-associated lymphoid tissue development: genetic evidence of synergism with lymphotoxin β . *J. Exp. Med.* 187:1977.
25. Mauri, D. N., R. Ebner, R. I. Montgomery, K. D. Kochel, T. C. Cheung, G. L. Yu, S. Ruben, M. Murphy, R. J. Eisenberg, G. H. Cohen, P. G. Spear, and C. F. Ware. 1998. LIGHT, a new member of the TNF superfamily, and lymphotoxin α are ligands for herpesvirus entry mediator. *Immunity* 8:21.
26. Sacca, R., C. A. Cuff, W. Lesslauer, and N. H. Ruddle. 1998. Differential activities of secreted lymphotoxin- α 3 and membrane lymphotoxin- α 1 β 2 in lymphotoxin-induced inflammation: critical role of TNF receptor 1 signaling. *J. Immunol.* 160:485.
27. Cuff, C. A., J. Schwartz, C. M. Bergman, K. S. Russell, J. R. Bender, and N. H. Ruddle. 1998. Lymphotoxin α 3 induces chemokines and adhesion molecules: insight into the role of LT α in inflammation and lymphoid organ development. *J. Immunol.* 161:6853.
28. Cuff, C. A., R. Sacca, and N. H. Ruddle. 1999. Differential induction of adhesion molecule and chemokine expression by LT α 3 and LT α 1 β 2 in inflammation elucidates potential mechanisms of mesenteric and peripheral lymph node development. *J. Immunol.* 162:5965.
29. Kratz, A., A. Campos-Neto, M. S. Hanson, and N. H. Ruddle. 1996. Chronic inflammation caused by lymphotoxin is lymphoid neogenesis. *J. Exp. Med.* 183:1461.
30. Sacca, R., S. Turley, L. Soong, I. Mellman, and N. H. Ruddle. 1997. Transgenic expression of lymphotoxin restores lymph nodes to lymphotoxin- α -deficient mice. *J. Immunol.* 159:4252.
31. Miyawaki, S., Y. Nakamura, H. Suzuka, M. Koba, R. Yasumizu, S. Ikehara, and Y. Shibata. 1994. A new mutation, aly, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *Eur. J. Immunol.* 24:429.
32. Shinkura, R., K. Kitada, F. Matsuda, K. Tashiro, K. Ikuta, M. Suzuki, K. Kogishi, T. Serikawa, and T. Honjo. 1999. Alymphoplasia is caused by a point mutation in the mouse gene encoding NF- κ B-inducing kinase. *Nat. Genet.* 22:74.
33. Malinin, N. L., M. P. Boldin, A. V. Kovalenko, and D. Wallach. 1997. MAP3K-related kinase involved in NF- κ B induction by TNF, CD95 and IL-1. *Nature* 385:540.
34. Kong, Y. Y., H. Yoshida, I. Sarosi, H. L. Tan, E. Timms, C. Capparelli, S. Morony, d. S. A. Oliveira, G. Van, A. Itie, et al. 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 397:315.
35. Wong, B. R., R. Josien, S. Y. Lee, B. Sauter, H. L. Li, R. M. Steinman, and Y. Choi. 1997. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J. Exp. Med.* 186:2075.
36. Anderson, D. M., E. Maraskovsky, W. L. Billingsley, W. C. Dougall, M. E. Tometsko, E. R. Roux, M. C. Teepe, R. F. DuBose, D. Cosman, and L. Galibert. 1997. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* 390:175.
37. Yasuda, H., N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, et al. 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. USA* 95:3597.
38. van den Berg, T. K., M. van der Ende, E. A. Dopp, G. Kraal, and C. D. Dijkstra. 1993. Localization of β 1 integrins and their extracellular ligands in human lymphoid tissues. *Am. J. Pathol.* 143:1098.
39. Sheehan, D., and B. Hrapchak. 1980. *Theory and Practice of Histotechnology*. Battelle Press, St. Louis, MO.
40. Anderson, A. O., and S. Shaw. 1993. T cell adhesion to endothelium: the FRC conduit system and other anatomic and molecular features which facilitate the adhesion cascade in lymph node. *Semin. Immunol.* 5:271.
41. Forster, R., A. E. Mattis, E. Kremmer, E. Wolf, G. Brem, and M. Lipp. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* 87:1037.
42. Ngo, V. N., H. Korner, M. D. Gunn, K. N. Schmidt, R. D. Sean, M. D. Cooper, J. L. Browning, J. D. Sedgwick, and J. G. Cyster. 1999. Lymphotoxin α β and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J. Exp. Med.* 189:403.
43. Pfeffer, K., T. Matsuyama, T. M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P. S. Ohashi, M. Kronen, and T. W. Mak. 1993. Mice deficient for the 55 kD tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell* 73:457.
44. Rothe, J., W. Lesslauer, H. Lotscher, Y. Lang, P. Koebel, F. Kontgen, A. Althage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann. 1993. Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature* 364:798.
45. Neumann, B., A. Luz, K. Pfeffer, and B. Holzmann. 1996. Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. *J. Exp. Med.* 184:259.