

A Role for Tumor Necrosis Factor Receptor Type 1 in Gut-associated Lymphoid Tissue Development: Genetic Evidence of Synergism with Lymphotoxin β

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Summary

Lymphotoxin α (LT α) signals via tumor necrosis factor receptors (TNFRs) as a homotrimer and via lymphotoxin β receptor (LT β R) as a heterotrimeric LT $\alpha_1\beta_2$ complex. LT α -deficient mice lack all lymph nodes (LNs) and Peyer's patches (PPs), and yet LT β -deficient mice and TNFR-deficient mice have cervical and mesenteric LN. We now show that mice made deficient in both LT β and TNFR type 1 (TNFR1) lack all LNs, revealing redundancy or synergism between TNFR1 and LT β , acting presumably via LT β R. A complete lack of only PPs in mice heterozygous for both *lt α* and *lt β* , but not *lt α* or *lt β* alone, suggests a similar two-ligand phenomenon in PP development and may explain the incomplete lack of PPs seen in *tnfr1*^{-/-} mice.

Key words: lymphotoxin beta • tumor necrosis factor receptor 1 • knockout mice • mesenteric lymph nodes • Peyer's patches

Studies on mice genetically deficient in various secondary lymphoid organs are increasing our understanding of the requirement or otherwise for these highly organized structures in immune function, from antiviral immunity (1) to autoimmunity (2). *Hox11*^{-/-} mice lack a spleen (3), whereas *aly/aly* mutant mice lack LNs and have a disorganized spleen (4, 5). Also, mice made deficient in the putative chemokine receptor BLR1 lack inguinal lymph nodes and fail to form primary B cell follicles in the spleen (6). Our studies have involved members of the TNF receptor and ligand families (7, 8). Studies of TNF family members are not only providing insight into the intricate microarchitecture of immune cell responses in lymphoid organs but also of chronic inflammatory states (9), such as the phenomenon termed lymphoid neogenesis (10).

TNF- α and TNF- β (lymphotoxin α ; LT α)¹ are the archetypal ligands of a growing family, which includes CD30 ligand (L), CD40L, FasL, TRAIL, and lymphotoxin β (LT β) (11, 12). LT β was discovered by virtue of its ability to anchor LT α to the cell surface, without which LT α is secreted as a homotrimer (LT α_3) (13, 14). LT α/β complex itself is a trimer with a predominant form (LT $\alpha_1\beta_2$) that binds LT β R, and a minor form (LT $\alpha_2\beta_1$) that binds TNF

receptor type 1 (TNFR1) (15–17). Both forms of LT α are produced by activated lymphocytes and NK cells (12, 18).

Historically, LT α_3 is known as a factor that causes cytotoxicity and inflammation, and signals via TNFR1 and TNFR2 (9, 19, 20). Although LT α/β complexes do not appear to mediate inflammation (21), pleiotropic effects of LT β R cross-linking are now emerging, including cytotoxicity (17, 22), chemokine induction (23), and integrin up-regulation (21). Studies with *lt α* ^{-/-} mice and *lt β* ^{-/-} mice are beginning to address the in vivo significance of these facets of LT α and LT β biology (2). However, initial studies of *lt α* ^{-/-} mice were dominated by the unexpected observation of a complete lack of LNs and Peyer's patches (PPs), as well as a disorganized spleen lacking follicular dendritic cells and germinal centers (24–27). Since mice deficient in TNFR have LNs, it had been assumed that the LT α/β complexes were responsible rather than LT α_3 . However, we recently showed that this explanation was not entirely correct (28). Specifically, we determined that *lt β* ^{-/-} mice retain mesenteric LNs (MLNs) and to a certain extent, cervical LNs, both of which drain mucosal surfaces. It was therefore a paradox that these LNs are absent in mice that lack the LT α_3 ligand and yet they are present in mice that lack the known receptors TNFR1 and TNFR2.

We now report that mice made deficient in both TNFR1 and LT β lack MLNs. We have thus revealed a redundancy or synergism between TNFR1 and LT β (presumably signaling via LT β R) that warrants further investi-

¹Abbreviations used in this paper: *tnfr*^{-/-}, mice deficient in both TNFR1 and TNFR2; LT, lymphotoxin; MLN, mesenteric lymph nodes; PPs, Peyer's patches.

gation in other aspects of TNFR1 and LT β biology. *Lt α ^{-/-}* mice and *lt β ^{-/-}* mice were derived as littermates by interbreeding, and unambiguously confirmed the lack of MLNs in *lt α ^{-/-}* mice and their presence in *lt β ^{-/-}* mice. Surprisingly, the latter studies also revealed a complete and specific lack of only PPs in *lt α ^{+/-}lt β ^{+/-}* mice. This presents a unique mouse model for the study of gastrointestinal immunology and suggests that two LT α ligands are involved in PP as well as MLN development, and may explain the incomplete lack of PPs seen in *tnfr1^{-/-}* mice.

Materials and Methods

Mice. *lt β ^{-/-}* and *lt β ^{+/+}* wild-type mice (expanded from original littermates of *lt β ^{-/-}* mice) are those described previously (28). A breeding pair of *lt α ^{-/-}* mice (24) was obtained from Nancy Ruddle (Yale University Department of Epidemiology and Public Health, Yale University), derived originally from David Chaplin (Washington University, St. Louis, MO). Mice deficient in both TNFR1 and TNFR2 (*dnfr2^{-/-}*) represent mice derived by interbreeding *tnfr1^{-/-}* mice with *tnfr2^{-/-}* mice (29). Various other knockout combinations were obtained by interbreeding. All mice were on a mixed background of C57BL/6 and 129/Sv. Breeding pairs of C57BL/6 Ly5.1 (CD45.1) mice were purchased from Clarence Reeder (Frederick Cancer Research Institute, Frederick, MD). All mice were maintained at Yale University in specific pathogen-free conditions. All procedures were conducted in accordance with Yale animal care and use guidelines.

LT β genotyping was by PCR using three oligonucleotides, yielding ~120- and 330-bp products for the *lt β ⁺* and *lt β ⁻* alleles, respectively. The oligonucleotide sequences are: LT β for, 5'-GAG-ACAGTCACACCTGTTG-3'; LT β rev, 5'-CCTGTAGTCCACCATGTCG-3'; and LT β neo, 5'-CTTGTTCAATGGCCGATCC-3'. TNFR1 and TNFR2 genotyping was by Southern blot analysis as described elsewhere (29).

Bone Marrow Chimeras. Hosts were exposed to 950 rads at 6–8 wk of age and, 1 d later, were given 2×10^6 total nucleated bone marrow cells intravenously in 0.2 ml of PBS. Bone marrow was from sex-matched 8–12-wk-old C57BL/6 Ly5.1 mice. 8 wk after irradiation, the relative ratio of CD45.1⁺ donor cells versus CD45.2⁺ host cells in peripheral blood was determined by flow cytometry. Both biotin-conjugated anti-CD45.1 and FITC-conjugated anti-CD45.2 were from PharMingen (San Diego, CA). The degree of chimerism was >95% in all cases. 9–10 wk after irradiation, recipients were challenged intraperitoneally with 0.1 mg of chicken γ globulin adsorbed to alum in 0.2 ml of PBS and were culled 12 d later.

Pathology. Visualization of brachial, axillary, inguinal, and popliteal LNs (30) was aided in some experiments by injecting 50 μ l of india ink into each footpad of the mice 3–4 h before culling. The prominence of PPs was greatly increased by immersing the intestine in 10% (vol/vol) acetic acid for 5 min before preservation in 10% neutral-buffered formalin. Hematoxylin and eosin staining was done on paraffin sections using standard procedures.

Immunohistology. Mice were challenged intraperitoneally at 6–8 wk of age with 0.1 mg of chicken γ globulin adsorbed to alum in 0.2 ml of PBS. Spleens and MLNs were harvested 12 d later and frozen in O.C.T. compound using a dry-ice/methylbutane bath. 5- μ m thick sections were cut onto silanized glass slides and fixed in cold acetone for 5 min before storage at -70°C. For staining, sections were allowed to thaw for 10 min and then rehy-

drated in PBS for 20 min. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide for 5 min and the sections were then washed with PBS for 10 min. Blocking was with PBS/3% BSA/0.1% (vol/vol) Tween 20 for 30 min. Staining for IgD used rat anti-mouse IgD (Southern Biotechnology Associates, Birmingham, AL), followed by biotin-conjugated goat anti-rat IgG (Southern Biotechnology Associates) and then β -galactosidase-conjugated avidin (Vector Laboratories, Burlingame, CA). Washing between layers was with PBS/0.1% (vol/vol) Tween 20 before reblocking as above. Germinal centers were stained using horseradish peroxidase-conjugated peanut agglutinin (EY Laboratories, San Mateo, CA; reference 31). IgM detection was with alkaline phosphatase-conjugated goat anti-mouse IgM (Southern Biotechnology Associates). Follicular dendritic cells were revealed with biotin-conjugated anticomplement receptor 1 (PharMingen, San Diego, CA; reference 32), followed by alkaline phosphatase-conjugated streptavidin (Zymed, South San Francisco, CA). Substrates for β -galactosidase, horseradish peroxidase, and alkaline phosphatase were HistoMark X-Gal (Kirkegaard and Perry Labs., Inc., Gaithersburg, MD), diaminobenzidine-brown (Zymed), and HistoMark Red (Kirkegaard and Perry Labs., Inc.), respectively.

Results

Lt α ^{-/-} Littermates of *lt β ^{-/-} Mice Lack MLNs.* Initial reports of the phenotype of two independently generated *lt α ^{-/-}* mouse strains differed in that one indicated that MLNs were absent (24), whereas the other indicated that lymphoid structures were present in the mesentery of 4 out of 14 mice (25). Most recently, among ~500 *lt α ^{-/-}* mice examined for MLNs, only 10 had a single MLN (33). It was thus suggested that the frequency of occurrence of MLNs in *lt α ^{-/-}* mice may vary depending on how the mice are housed (33). If true, this would perhaps apply equally to *lt β ^{-/-}* mice, which we described as consistently having MLNs (28). Furthermore, Alimzhanov et al. independently generated *lt β ^{-/-}* mice and found that only ~75% of these mice have MLNs (34). It was therefore also conceivable that there are effects of background genes, although all mice examined were on a mixed background of 129/Sv and C57BL/6. The studies here were begun to examine these issues and determine why *lt β ^{-/-}* mice have MLNs despite the fact that *lt α ^{-/-}* mice mostly do not.

The *lt α* and *lt β* genes are separated by only ~6 kbp in the MHC locus (12). Thus, we reasoned that it would be possible to generate *lt α ^{-/-}* mice and *lt β ^{-/-}* mice as littermates by interbreeding mice which are heterozygous for both *lt α* and *lt β* (*lt α ^{+/-}lt β ^{+/-}* mice). In this way, 137 progeny were generated and genotyped as described in Materials and Methods. *Lt α ^{-/-}*, *lt β ^{-/-}*, and *lt α ^{+/-}lt β ^{+/-}* mice occurred in a relatively normal Mendelian fashion ($n = 31$, 40, and 66, respectively). Some of these mice were examined at 6–8 wk of age. *Lt α ^{-/-}* mice did not have MLNs ($n = 14$), whereas almost all of their *lt β ^{-/-}* littermates did ($n = 25$). A single *lt β ^{-/-}* mouse out of 25 appeared to lack MLNs.

Lymphotoxin Gene Dosage Effect in PP Development. Unlike *lt α ^{-/-}* and *lt β ^{-/-}* mice, the above heterozygous *lt α ^{+/-}lt β ^{+/-}* mice had all LNs ($n = 30$), except that two

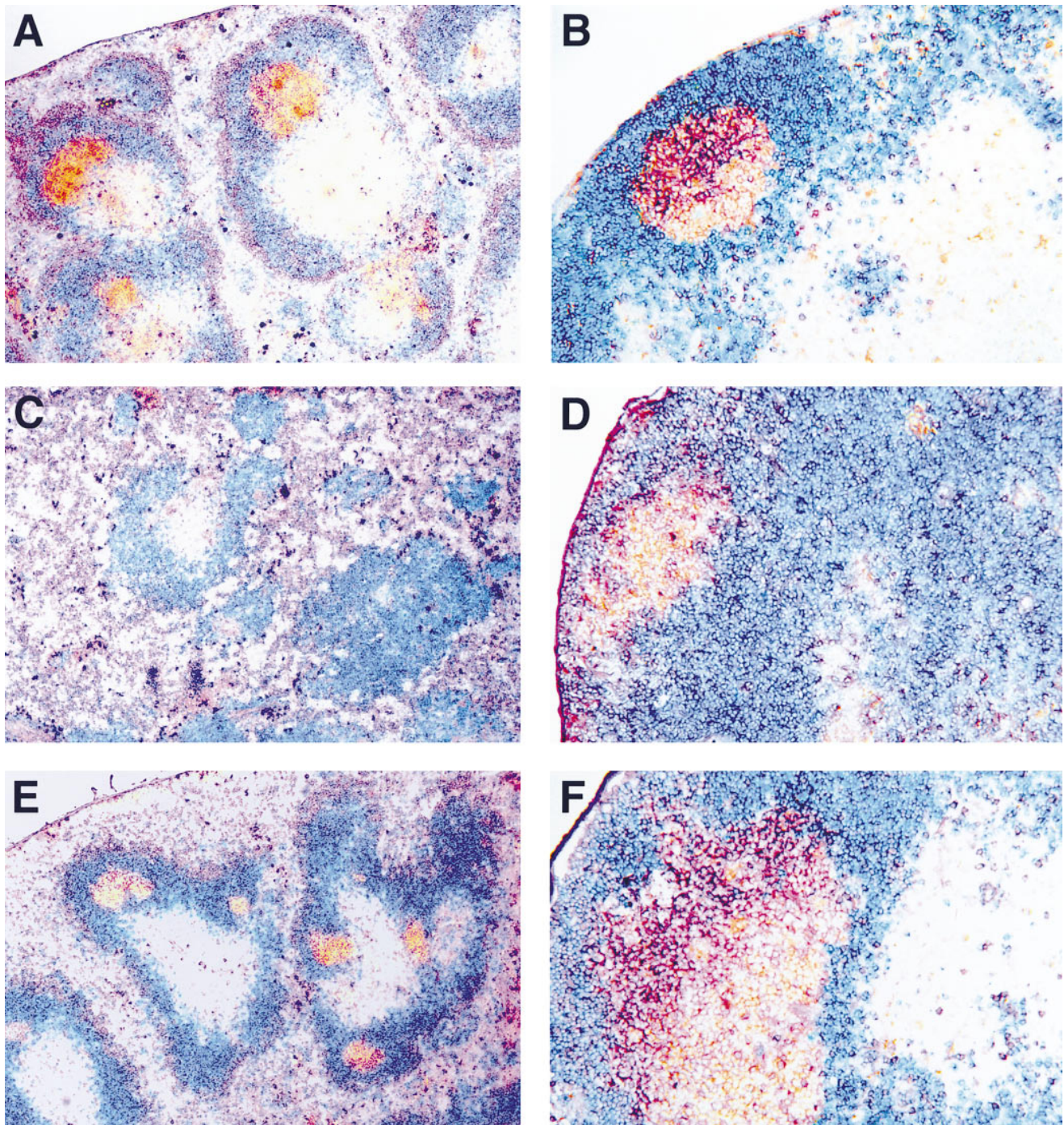


Figure 1. $lt\alpha^{+/-}lt\beta^{+/-}$ mice have relatively normal lymphoid organ architecture. Mice were challenged intraperitoneally with 0.1 mg of chicken γ globulin adsorbed to alum and culled 12 d later. Spleen (A, C, and E) and MLNs (B, D, and F) sections were stained for IgM (red), IgD (blue) and peanut agglutinin-binding germinal centers (brown). A and B, wild-type; C and D, $lt\beta^{-/-}$; E and F, $lt\alpha^{+/-}lt\beta^{+/-}$. Original magnification, $\times 65$ and $\times 150$ for spleen and MLN, respectively.

mice had only one inguinal LN and one mouse had none. Surprisingly, however, $lt\alpha^{+/-}lt\beta^{+/-}$ mice showed a complete lack of PPs ($n = 30$), whereas both $lt\alpha^{+/-}$ mice ($n = 13$) and $lt\beta^{+/-}$ mice ($n = 14$) have PPs as well as all LNs. Having made this observation, we examined $lt\alpha^{+/-}lt\beta^{+/-}$ mice further. At 6–8 wk of age, the gross spleen architec-

ture was normal by hematoxylin and eosin histology (data not shown). Immunohistology for complement receptor 1 in the spleen (done as previously described; reference 28) revealed the presence of follicular dendritic cells (data not shown). Also, splenic germinal centers were formed in discrete B cell follicles after intraperitoneal challenge, except there

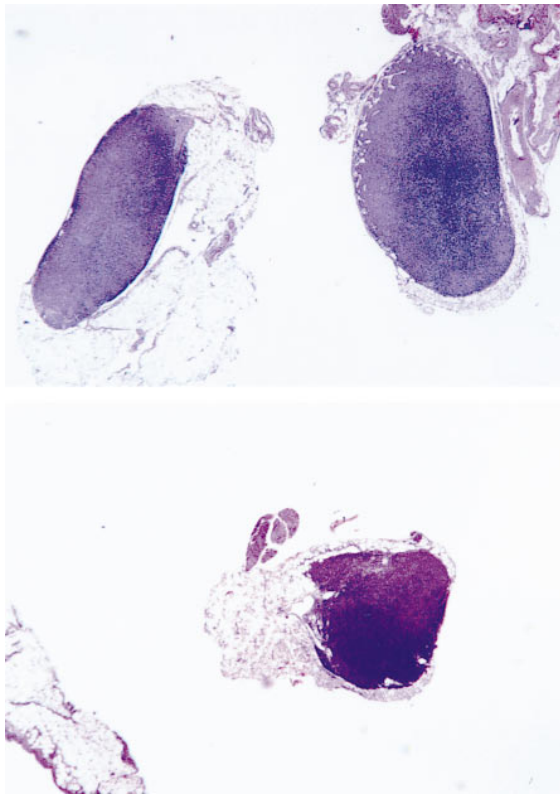


Figure 2. $Lt\beta^{-/-}tnfr1^{+/+}$ mice have defective MLN development. $Lt\beta^{-/-}tnfr1^{-/-}$ mice completely lack MLNs and $Lt\beta^{-/-}tnfr1^{+/+}$ littermates have MLN of apparently normal size and number (top), but $Lt\beta^{-/-}tnfr1^{+/+}$ littermates most often have only a single, small MLN (bottom). Hematoxylin and eosin histology; original magnification: $\times 15$.

appeared to be some disorganization among $IgM^{+}IgD^{lo/-}$ marginal zone B cells (Fig. 1 E). $Lt\alpha^{-/-}$ mice (24–28) and $Lt\beta^{-/-}$ mice (28, 34) have severe defects in all of these aspects of lymphoid organogenesis.

The organization of the MLNs of $Lt\alpha^{+/-}Lt\beta^{+/-}$ mice was also relatively normal (Fig. 1 F). As previously noted (28), the organization of the MLNs of $Lt\beta^{-/-}$ mice is not normal in that there appears to be a generalized B cell infiltration, but B cell follicles are found around the rim of MLNs and germinal center B cell clusters are formed despite the absence of follicular dendritic cells (reference 28; Fig. 1 D).

The lack of PPs in $Lt\alpha^{+/-}Lt\beta^{+/-}$ mice was confirmed in progeny from intercrossing $Lt\alpha^{-/-}$ mice with $Lt\beta^{-/-}$ mice ($n = 4$). Bone marrow chimeras were also generated using wild-type bone marrow, to examine whether or not the lack of PPs was reversible. None of the $Lt\alpha^{+/-}Lt\beta^{+/-}$ recipients showed any sign of PPs 10–12 wk after irradiation, but they did have LNs ($n = 9$). None of the $Lt\alpha^{-/-}$ recipients had MLNs ($n = 8$), but all of the $Lt\beta^{-/-}$ recipients did ($n = 11$). None of the $Lt\alpha^{-/-}$ recipients or $Lt\beta^{-/-}$ recipients had PPs. $Lt\beta^{+/+}$ wild-type recipients had MLNs and PPs ($n = 4$).

TNFR1 Is Involved in MLN Development. Both $Lt\beta^{-/-}$ mice and $dtufr^{-/-}$ mice have MLNs, and yet $Lt\alpha^{-/-}$ mice do not. This led us to propose that $LT\alpha$ may act without $LT\beta$ (i.e., as $LT\alpha_3$) via an as yet unidentified receptor (28). To

test this hypothesis, we generated mice lacking both $LT\beta$ and TNFR and examined them for the presence of MLNs. Since TNFR-deficient mice were originally obtained as $dtufr^{-/-}$ mice, the first mice generated here were $Lt\beta^{-/-}dtufr^{-/-}$ mice. At 6–8 wk of age, $Lt\beta^{-/-}dtufr^{-/-}$ mice showed a complete lack of MLNs ($n = 10$), whereas $Lt\beta^{+/-}dtufr^{-/-}$ mice still had MLNs ($n = 5$).

In a similar way to $Lt\alpha^{-/-}$ mice, it is conceivable that the apparent absence of MLNs in $Lt\beta^{-/-}dtufr^{-/-}$ mice is due to a possible lack of immune competence and/or lymphocyte homing, and that this might be reversed after reconstitution with wild-type bone marrow. We therefore generated wild-type bone marrow chimeras. However, none of the bone marrow chimeras had MLNs 10–12 wk after reconstitution ($n = 11$).

In the meantime, we also generated $Lt\beta^{-/-}tnfr1^{-/-}$ and $Lt\beta^{-/-}tnfr2^{-/-}$ mice. The latter had MLNs ($n = 4$) but $Lt\beta^{-/-}tnfr1^{-/-}$ mice clearly did not ($n = 5$). Most $Lt\beta^{-/-}tnfr1^{+/+}$ littermates ($n = 5$) had one small MLN (Fig. 2). One $Lt\beta^{-/-}tnfr1^{+/+}$ littermate did not appear to have MLNs, whereas another had two small MLNs. This may be explained by the fact that $tnfr1$ heterozygosity is known to result in a partial phenotype at least in some respects (35), but at the same time $Lt\beta^{+/-}tnfr1^{+/+}$ mice had MLNs of a normal size ($n = 13$).

Discussion

The study reported here extends our knowledge of the roles of TNF ligand/receptor family members in lymphoid organogenesis (Table 1). Based on several observations, we had previously hypothesized that both TNFR1 and $LT\beta R$ may be involved in PP development (28). First, both $Lt\alpha^{-/-}$ mice (24, 25) and $Lt\beta^{-/-}$ mice (28, 34) completely lack PPs. Second, Rennert et al. observed a complete lack of PPs in mice administered recombinant soluble $LT\beta R$ in utero (36). Third, $tnfr1^{-/-}$ mice lack PPs but have reduced numbers of residual lymphoid aggregates (37). Defective PP development was also reported recently with an independently generated $tnfr1^{-/-}$ mouse strain (29). Others reported that $tnfr1^{-/-}$ mice have PPs but that they appear flattened due to a lack of B cell follicle structures (38). However, even this study noted that $tnfr1^{-/-}$ mice have on average only two to four such PPs compared with six to eight PPs in the wild-type control mice (38).

In this study, we show the existence of a gene dosage effect that is consistent with a role for both TNFR1 and $LT\beta R$ in PP development. That is, $Lt\alpha^{+/-}Lt\beta^{+/-}$ mice specifically lack only PPs, but $Lt\alpha^{+/-}$ mice and $Lt\beta^{+/-}$ mice do not. If $LT\alpha$ and $LT\beta$ form a single species that signals via a single receptor, it might be expected that either $LT\alpha$ or $LT\beta$ would be the limiting factor and that heterozygosity in either $Lt\alpha$ or $Lt\beta$ alone should result in the lack of PPs seen in $Lt\alpha^{+/-}Lt\beta^{+/-}$ mice. However, this is not the case. Only when both $Lt\alpha$ and $Lt\beta$ are heterozygous does insufficiency become evident. One interpretation would be that two ligands (e.g., $LT\alpha_3$ and $LT\alpha_{1\beta_2}$ signaling via TNFR1

Table 1. Phenotypes of Mice Made Genetically Deficient in TNF Ligand/Receptor Family Members

Family Members	<i>ltα</i> ^{-/-}	<i>ltβ</i> ^{-/-}	<i>tnfα</i> ^{-/-}	<i>tnfr1</i> ^{-/-}	<i>ltα</i> ^{+/-} <i>ltβ</i> ^{+/-}	<i>ltβ</i> ^{-/-} <i>tnfr1</i> ^{-/-}
Spleen						
Primary B cell follicles	-	-	-	-	+	ND
Marginal zone	-	-	+	+	+/-	ND
Germinal centers	-	-	-	-	+	ND
Follicular dendritic cells	-	-	-	-	+	ND
MLNs						
Primary B cell follicles	-	+/-	ND	-	+	-
Germinal centers	-	+/-	ND	-	+	-
Follicular dendritic cells	-	-	ND	-	+	-
Cervical LN	-	+/-	+	+	+	-
Inguinal LN	-	-	+	+	+/-	-
Other LN	-	-	+	+	+	-
PPs	-	-	+/-	+/-	-	-

This table is based on citations in the text and others as summarized elsewhere (8), as well as the findings from this study. Unlike *ltα*^{+/-}*ltβ*^{+/-} double heterozygous mice, *ltα*^{+/-} mice and *ltβ*^{+/-} mice have PPs as well as all LNs. Other features of the phenotype of *ltα*^{+/-} mice and *ltβ*^{+/-} mice have not been determined. Also, although *ltβ*^{-/-}*tnfr1*^{-/-} mice do not have MLNs, *ltβ*^{-/-}*tnfr2*^{-/-} mice do.

and LTβR, respectively) are involved in PP development, and that heterozygosity in either one or the other alone is not enough to cause a complete loss of PP development. This two-receptor model might therefore provide an explanation for the partial defect in PP development seen in *tnfr1*^{-/-} mice.

Clearly, our results show that both TNFR1 and LTβ are involved in MLN development, even though both *tnfr1*^{-/-} mice and *ltβ*^{-/-} mice have MLNs. TNFR1 also functions independently of TNFR2 in this regard, as *ltβ*^{-/-}*tnfr2*^{-/-} mice still have MLNs. We have thus revealed a previously unappreciated relationship between TNFR1 and LTβ (presumably acting via LTβR). An explanation for the lack of MLN in *ltα*^{-/-} mice might therefore be that LTα deficiency actually eliminates both ligands of the relationship (i.e., LTα₃ and LTα₁β₂ signaling via TNFR1 and LTβR, respectively). LTα₃ itself is not believed to bind LTβR (16, 17).

However, having said this, it has been indicated that *ltβ*^{-/-} mice lack MLNs (34). Thus, the relationship between TNFR1 and LTβR may be one of synergism with LTβR as the dominant partner. At the same time, the presence of MLN in *ltβ*^{-/-} mice would imply that LTβR has a ligand besides the LTα/β complex. Indeed, Mauri et al. have very recently described a new LTβR ligand (LIGHT) as well as a new LTα₃ receptor, the herpesvirus entry mediator, expressed by lymphocytes (39).

The molecular basis for the relationship between TNFR1 and LTβ (presumably via LTβR) remains to be determined. It is conceivable that TNFR1 and LTβR signaling in MLN development is simultaneous and that they interact at the level of intracellular signal transducers. Certainly, activation of LTβR has been shown to potentiate TNF-α

cytotoxicity, possibly reflecting cross-talk between signaling pathways (17, 22). Ligation of LTβR causes recruitment of TNFR-associated factor family members (40–42), and activation of NF-κB and cell death by distinct signaling pathways (42, 43).

Thus far, our studies of *ltβ*^{-/-} mice have evaluated the defects in lymphoid organogenesis (reference 28 and this study). We are now beginning to examine whether or not LTβ has roles in vivo in other respects. Certainly, in vitro studies have shown that signaling via LTβR causes cytotoxicity to some cell lines (17, 22), chemokine expression (23), and integrin upregulation (21). It remains to be seen whether or not the relationship between TNFR1 and LTβ (presumably via LTβR) in gut-associated lymphoid tissue development extends to any other facets of biology. With this in mind, caution is advised when interpreting the in vivo role (or rather, apparent lack thereof) of LTβ and TNFR1 based on studies of *ltβ*^{-/-} mice and *tnfr1*^{-/-} mice alone.

Finally, *ltα*^{+/-}*ltβ*^{+/-} mice may prove to be a useful PP-less mouse model, not only for the study of gastrointestinal infection, but also of oral tolerance, oral vaccination, and chronic disorders such as inflammatory bowel disease (44–46). *ltα*^{+/-}*ltβ*^{+/-} mice are being further characterized, particularly with respect to the subtle defect observed in splenic marginal zone organization. Although it remains possible that *ltα*^{+/-}*ltβ*^{+/-} mice have other as yet unidentified defects, unlike any other previously described mouse, these mice specifically and completely lack only PPs and do not appear to have any of the major abnormalities associated with *ltα*^{-/-} and *ltβ*^{-/-} mice.

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