# Regulation of Peripheral Lymph Node Genesis by the Tumor Necrosis Factor Family Member TRANCE

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## Abstract

Proper lymph node (LN) development requires tumor necrosis factor–related activation-induced cytokine (TRANCE) expression. Here we demonstrate that the defective LN development in TRANCE$^{-/-}$ mice correlates with a significant reduction in lymphotoxin (LT)$\alpha\beta^+\alpha_4\beta_7{}^+$CD45$^+$CD4$^+$CD3$^-$ cells and their failure to form clusters in rudimentary mesenteric LNs. Transgenic TRANCE overexpression in TRANCE$^{-/-}$ mice results in selective restoration of this cell population into clusters, and results in full LN development. Transgenic TRANCE-mediated restoration of LN development requires LT$\alpha\beta$ expression on CD45$^+$ CD4$^+$CD3$^-$ cells, as LNs could not be induced in LT$\alpha^{-/-}$ mice. LT$\alpha^{-/-}$ mice also showed defects in the fate of CD45$^+$CD4$^+$CD3$^-$ cells similar to TRANCE$^{-/-}$ mice. Thus, we propose that both TRANCE and LT$\alpha\beta$ regulate the colonization and cluster formation by CD45$^+$ CD4$^+$CD3$^-$ cells in developing LNs, the degree of which appears to correlate with the state of LN organogenesis.

Key words: TRANCE • lymphotoxin • tumor necrosis factor • lymph node • organogenesis

## Introduction

LNs are highly organized structures found in higher vertebrates, which concentrate antigens, antigen-presenting cells, and antigen-specific lymphocytes into physical proximity to provide microenvironments for optimal immune responses. In addition, within this highly organized structure, antigen-specific T or B lymphocytes are activated and differentiate to become effector cells. Therefore, LNs are believed to be critical secondary lymphoid organs for the initiation of immune responses.

LN development is a continuous process, but five distinct stages of LN organogenesis can be proposed based on histological studies (1–4).

*Stage I.* The lymphatic system develops by the formation of lymphatic sacs that originate as endothelial budding from the veins (starting as early as embryonic day [E]10.5 of the mouse). Prox1, a homologue of *Drosophila prospero*, was recently shown to be essential for budding of endothelial cells from veins, the deficiency of which resulted in complete arrest of the lymphatic system and subsequent early lethality (5).

*Stage II.* Lymphatic vessels develop by endothelial sprouting from those lymph sacs.

*Stage III.* The anlagen of LNs are formed by invagination of mesenchymal connective tissues into the lumen of growing lymph sacs. The resulting network of lymph channels forms the lymphatic plexus, which can give rise to LNs. Mesenchymal connective tissue invaginations are thus the anlage of LN parenchyma, which contain reticular cells, fibroblasts, some leukocytes, capillaries, and vascular loops. Therefore, primordial LNs are primarily cellular reticula surrounded by endothelial cells from lymph channels, which will form the subcapsular sinus. The blood supply of the fetal LNs is already present at this stage, via vessels that

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run through the stalk of the connective tissue invagination. It is within the LN anlage that the specialized endothelial cells form, supporting leukocyte traffic from the bloodstream into the underlying LN parenchyma.

*Stage IV.* The cellular density and leukocyte content in the LN anlage increase and the subcapsular sinus is formed. It is probably during this stage of LN formation that the majority of hematopoietic lineage precursor cells colonize the nodal parenchyme.

*Stage V.* The anlage of LNs continues to expand and form capsules. The cellular density and leukocyte content continue to increase, and mature lymphocytes migrate into the developing LNs and establish proper cellular microarchitecture.

Recent genetic experiments have revealed that members of the TNF/TNFR family are critically involved in LN organogenesis and in the maintenance of LN and splenic microarchitecture (6). Inactivation of the lymphotoxin (LT)[1] pathway in mice resulted in the absence of LNs and Peyer's patches (PPs), indicating the pivotal role of membrane LT ($LT\alpha\beta$) and its receptor (LT-βR) for LN genesis (7–14). Histological analysis of LN regions in mutant adult mice showed no LN-like structures but retained intact lymphatic vasculature of vein, arteria, and lymphatic vessel (14), suggesting that defects in these mice do not lie in the formation of lymphatic vasculature (stage II). In addition to its role during the LN genesis, the $LT\alpha\beta$/LT-βR system is also critical for the maintenance of microarchitecture of adult spleens, as antagonism of $LT\alpha\beta$ in adult mice caused disruptions in splenic T/B organization, marginal zone integrity, and follicular dendritic cell networks (11, 13–16).

The TRANCE/TRANCE-R pathway provided a second example of TNF/TNFR family members critically involved in LN development. Inactivation of murine TRANCE or TRANCE-R resulted in the absence of all peripheral LNs (PLNs) and mesenteric LNs (MLNs) (17, 18). However, in contrast to the $LT\alpha\beta$/LT-βR system, the TRANCE/TRANCE-R system was not essential for the genesis of PPs. In addition, it was reported that disruption of the TRANCE/TRANCE-R system did not affect splenic microarchitecture (17).

Despite accumulating data implicating various TNF/TNFR family members (6), and also their signal transducers such as TNFR-associated factor (TRAF)6, nuclear factor (NF)-κB–inducing kinase (NIK), or RelB, in the regulation of LN development (19–23), little is known about how these molecules regulate LN genesis during development. Hence, we generated $TRANCE^{-/-}$ mice to further dissect the role of TRANCE during LN development and also to study the potential relationship between the TRANCE/TRANCE-R and the LT/LT-βR systems in the regulation of LN genesis. Here we show that arrested LN development in $TRANCE^{-/-}$ mice is due to the impaired colonization and subsequent cluster formation of hematolymphoid precursor cells within the developing LN anlage. In particular, we show that the degree of colonization and cluster formation of $\alpha_4\beta_7{}^+CD4^+CD3^-$ cells in the primordial LN structures correlated with the presence of LNs in adults. Similar to $TRANCE^{-/-}$ mice, $LT\alpha^{-/-}$ mice also showed impaired colonization and cluster formation of $\alpha_4\beta_7{}^+CD4^+CD3^-$ cells in the LN anlage. These results thus suggest that both TRANCE and LT ligands regulate the LN genesis by controlling the colonization and cluster formation of $\alpha_4\beta_7{}^+CD4^+CD3^-$ cells during LN development. As the defects in the TRANCE/TRANCE-R or LT/LT-βR system did not affect the initiation of colonization by $\alpha_4\beta_7{}^+CD4^+CD3^-$ cells, the results suggest that neither TRANCE nor LT are intrinsically required for the initiation of the formation of LN anlage, but are required for further development during stage III of the LN anlage.

## Materials and Methods

*Generation of TRANCE-deficient or Transgenic Mice.* To generate $TRANCE^{-/-}$ mice, a 129SvJ mouse λ FIXII genomic library (Stratagene) was screened using the full-length murine TRANCE cDNA probe. An ~42-kb contig was assembled from overlapping clones comprising exons 2–5, which allowed construction of the replacement vector, pPNT-TRANCE-RV1.2, as follows: a 1.8-kb BamHI-KpnI genomic short arm subcloned into the pSL301 vector (Invitrogen) was released as an EcoRI-KpnI fragment for antisense insertion 5′ to PGKneopA of the pPNT vector. An 8.2-kb SspI-SspI genomic long arm subcloned into the HincII site of pBS $KSII^+$ (Stratagene) was released as an XhoI-NotI fragment for 3′ antisense ligation next to PGKneopA. E14.1 embryonic stem cells ($5 \times 10^7$) were electroporated with NotI-linearized pPNT-TRANCE-RV1.2 and transformants were selected after 7–8 d in 180 μg/ml active geneticin (GIBCO BRL) plus 2 μM gancyclovir (Roche), the latter yielding about threefold enrichment. Genomic DNAs isolated from individual colonies were EcoRI digested and Southern blot hybridized with a 5′ flanking probe (0.95-kb EcoRI-BamHI fragment immediately adjacent to the short arm). Two targeted embryonic stem cell clones (TR-73 and TR-96) carrying single integration events, as shown by Southern probing with a 1.6-kb NotI-KpnI PGKNeopA fragment, were microinjected into C57BL/6 blastocysts to produce germline chimeras.

For TRANCE.TG mice, we have generated several transgenic mouse lines overexpressing TRANCE under the control of the murine CD4 enhancer/promoter lacking CD4 silencer sequences and thus driving the expression of transgene in CD4 as well as in CD8 cells (24; Wong, B.R, and Y. Choi, unpublished data). Three independent transgenic lines were obtained. Two lines (TRANCE.TG-3, TRANCE.TG-40) expressed transgenic TRANCE only on T cells, but one line, TRANCE.TG-1, expressed transgenic TRANCE in both B and T cells as shown in this study. None of the mice expressed detectable levels of transgenic TRANCE on $CD45^+CD4^+CD3^-$ cells in developing LNs. These results suggest that promoter/enhancer elements for CD4 expression in $CD45^+CD4^+CD3^-$ cells and T cells must be different.

*PCR Analyses.* To genotype the mice, genomic PCR was performed on phenol/chloroform-extracted tail biopsy DNA or

[1]*Abbreviations used in this paper:* CLN, cervical LN; GC, germinal center; HPRT, 5′-hypoxanthine ribosyltransferase; LT, lymphotoxin; MAdCAM, mucosal addressin cell adhesion molecule; MLN, mesenteric LN; PLN, peripheral LN; PP, Peyer's patch; rMLN; rudiments of MLN; TRANCE, TNF-related activation-induced cytokine; WT, wild-type.

fetal liver genomic DNA in a 50-μl PCR reaction (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 0.5 U Taq polymerase; Sigma-Aldrich). Primer composition and concentration, together with the product size and PCR conditions used, were the following: 5′-TRANCE (0.5 μM), 5′-CCAAGTAGTGGATTCTAAATCCTG-3′; 3′WT (0.2 μM), 5′-GGTTGGACACCTGAATGCTAATTTC-3′ (345 bp); and 3′ NeoRec (0.2 μM), 5′-ATTCGCAGCGCATCGCCTTC-TATCG-3′ (575 bp); 94°C, 5 min; 30 cycles (94°C, 30 s; 58°C, 45 s, 72°C, 1 min); and 72°C, 7 min.

Semiquantitative reverse transcription PCR was performed on first-strand synthesized thymocyte cDNA as described (25). Primers used were the following: 5′-hypoxanthine ribosyltransferase (HPRT), 5′-GTAATGATCAGTCAACGGGGGAC-3′; 3′-HPRT, 5′-CCAGCAAGCTTGCAACCTTAACCA-3′; 5′-TRANCE-R, 5′-CCCGAATTCTACTACAGGAAGGGA-GGGAAG-3′; 3′-TRANCE-R, 5′-CCTGCTGGATTAGGA-GCAGTG-3′; 5′-TRANCE, 5′-CCTGAGACTCCATGAAA-CGC-3′; and 3′-TRANCE, 5′-TAACCCTTAGTTTTCC-GTTGC-3′.

*LN Enumeration.* Video-captured binocular stereomicroscope images (×10 magnification) were obtained during inspection under a Leica M16 dissection microscope. In addition, some mice were injected subcutaneously with Evan's blue dye (1:10 dilution) 3 h before killing.

*Abs and Flow Cytometry.* The following reagents and Abs were used for staining: TRANCE-R-$hIgG_1$ (TR-Fc [25]); 1E6.66 (rat anti–TRANCE-R mAb) GK5.1 (anti-CD4); RM4-5 (anti-CD4); 145-2C11 (anti-CD3); 53-6.7 (anti-CD8); 104 (anti-CD45.2); H57-597 (anti-TCR); RA3-6B2 (anti-B220); R6-60.2 (anti-IgM); 11-26C.2a (anti-IgD); HL-3 (anti-CD11c); M1/70 (anti-CD11b); PC61 (anti-CD25); IM7 (anti-CD44); S7 (anti-CD43); mLT-βR-$hIgG_1$ (11); AF.H6 (agonistic anti–mLT-βR mAb [13]; DATK-32 (anti–LPAM-1); MECA-367 (anti–mucosal addressin cell adhesion molecule [MAdCAM]-1); and 3D6.112 (antisialoadhesin; Serotec). Samples were erythrocyte lysed and pretreated with anti-CD16/32 FcR mAb 2.4G2 (1:50 dilution) before undergoing four-color fluorometry using a FACSCalibur™ with argon and helium–neon lasers (Becton Dickinson).

*Immunofluorescence Analysis.* For immunofluorescence analyses on frozen sections, mouse organs were harvested, frozen, sectioned, and, after being preblocked with 2.4G2 in PBS, stained as described (11, 12, 26). In brief, cryostat sections (5 μm thick) were allowed to air dry and fixed in acetone. The sections were incubated with primary Abs for 45 min, rinsed in PBS, and incubated with secondary Abs for 30 min, rinsed with PBS, and coverslipped using antifading mounting media. The primary Abs used were Alexa-488–conjugated anti-CD4 and biotin-labeled anti–MAdCAM-1. For whole-mount immunofluorescence analysis, freshly isolated day 0 MLNs or rudiments of MLNs (rMLNs) were stained with FITC-conjugated anti-CD4 Ab and PE-labeled anti–MAdCAM-1. For this, the whole MLNs or rMLNs were incubated with Abs at 4°C for 3–4 h, washed three times, and visual data were acquired with an Axioplan 2 fluorescent microscope (ZEISS) equipped with a SensiCam CCD camera (Cooke Corporation) using SlideBook software (Intelligent Imaging Co.).

*Germinal Center Formation.* 6-wk-old mice were immunized intraperitoneally with 50 μg of (4-hydroxy-3-nitrophenyl-acetyl)-KLH ($NP_{12}$-KLH; Biosearch Technologies). Splenic germinal center (GC) formation was examined 10 d after immunization in cryosections with peanut agglutinin (PNA)-biotin/streptavidin–PE and IgM-FITC as described (11, 12).

## Results

*Inactivation of the Murine TRANCE Gene: A Gene Dosage Effect on the Level of TRANCE Expression.* $TRANCE^{-/-}$ mice were described as having a defect in LN organogenesis but not in PP development or maintenance of splenic architecture (17). To further study the role of TRANCE in LN genesis, we have generated $TRANCE^{-/-}$ mice by homologous recombination, in which nucelotides 699–1089 of exon 5 (amino acids 185–316) plus the additional 1123-bp 3′ untranslated region have been deleted (data not shown). The overall phenotype of our $TRANCE^{-/-}$ mice was essentially similar to that described (17) such that $TRANCE^{-/-}$ mice failed to develop PLNs and MLNs (see below) and osteoclasts (data not shown). Of note, however, we showed here that $TRANCE^{+/-}$ mice displayed intermediate levels of TRANCE expression (Fig. 1), indicating a gene dosage effect.

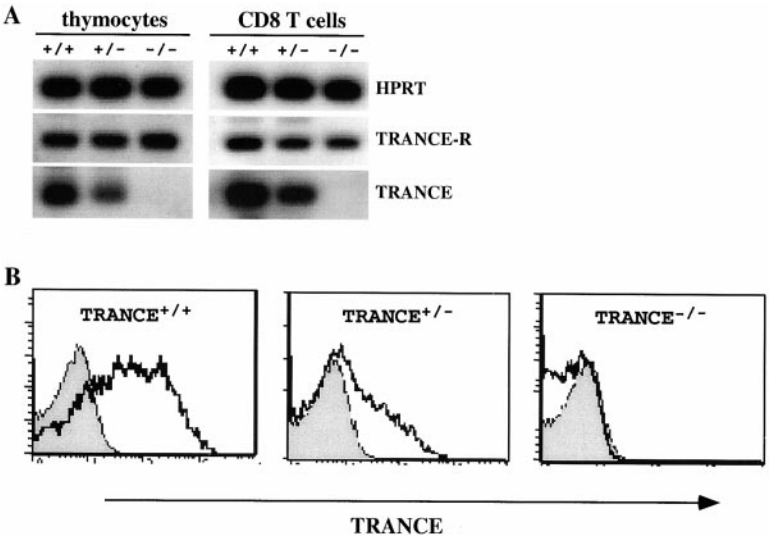



**Figure 1.** Generation of $TRANCE^{-/-}$ mice: a gene dosage effect on TRANCE expression. (A) Reverse transcription PCR analysis of thymocytes or activated $CD8^+$ T cells from WT, $TRANCE^{+/-}$, and $TRANCE^{-/-}$ mice. TRANCE-R mRNA expression was also examined and HPRT served as an internal control. (B) Flow cytometric analysis of activated $CD8^+$ T cells from WT, $TRANCE^{+/-}$, and $TRANCE^{-/-}$ mice. Cells were stained with $hIgG_1$ (gray lines) or TRANCE-R-$IgG_1$ (thick lines) followed by PE-coupled goat anti–human $IgG_1$.

*Altered Splenic B Cell Follicles in TRANCE$^{-/-}$ Mice.* There were no major defects in overall splenic architecture in TRANCE$^{-/-}$ mice, as reported (17). However, immunofluorescence analyses of spleen sections revealed altered splenic microarchitecture in >70% of TRANCE$^{-/-}$ mice. Although lymphocytes segregated into discrete T and B cell zones, there were defects in B cell follicle formation and marginal zone integrity. The degree of these defects in TRANCE$^{-/-}$ mice, however, varied considerably even in the same mice: we observed T cell–rich periarteriolar lymphoid sheath with only sparse B cell rings but no follicles, with B cell rings but no follicles, or with apparently normal B cell follicles (Fig. 2 A). To assess marginal zone integrity, we stained spleen sections with Ab specific for sialoadhesin, a marker of marginal zone macrophages. Wild-type (WT) and TRANCE$^{+/-}$ mice revealed a tight, bright ring of staining which encompassed the entire marginal zone, broken only by bridging channels (data not shown). In contrast, most TRANCE$^{-/-}$ mice had variable staining which was repeatedly interrupted and diffuse. About 75% of TRANCE$^{-/-}$ mice manifested a defect in marginal zone integrity; the remaining ~25% appeared relatively normal. TRANCE$^{-/-}$ mice, which had relatively normal marginal zone staining, also had relatively intact B cell follicles (data not shown). Despite these defects, upon immunization with the T cell–dependent antigen nitrophenol-haptenated KLH, GCs were formed in spleens of TRANCE$^{-/-}$ mice that were comparable to those in WT mice (Fig. 2 B). These results suggest that TRANCE contributes to but is not essential for the proper formation of B cell follicles in spleen.

*Absence of LNs in TRANCE$^{-/-}$ Mice.* TRANCE$^{-/-}$ mice showed defective LN development (Fig. 3 A) but normal PPs (data not shown), consistent with a previous report (17). Although most TRANCE$^{-/-}$ mice failed to develop any PLNs or MLNs, we have occasionally observed that cervical LNs (CLNs) develop in TRANCE$^{-/-}$ mice (~30% of TRANCE$^{-/-}$ mice examined contained one of the CLNs; Fig. 3 B). Such CLNs appeared to be bona fide LNs with T and B cells segregated into discrete zones (Fig. 3 B). However, B cells failed to form follicles in those CLNs, similar to the defect observed in the spleens of ~70% of TRANCE$^{-/-}$ mice.

We also noted that a small number of the TRANCE$^{+/-}$ mice, having an intermediate level of TRANCE expression, failed to develop some PLNs (~7% of TRANCE$^{+/-}$ mice lacked one inguinal LN and ~3% of TRANCE$^{+/-}$ mice lacked one iliac LN; Fig. 3 C). However, none of the TRANCE$^{+/-}$ mice showed any abnormality in the formation of MLNs. Thus, these results indicate a haploinsufficiency effect of TRANCE on the formation of some PLNs.

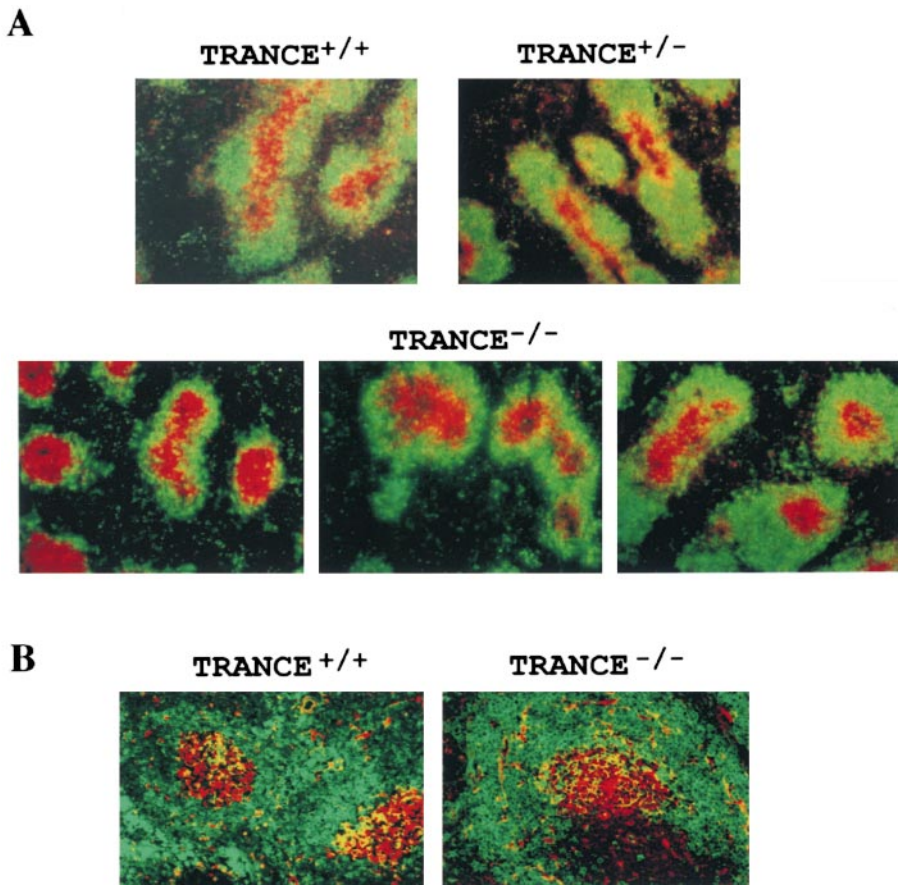



**Figure 2.** Altered primary B cell follicles but apparently normal formation of GCs in spleen of TRANCE$^{-/-}$ mice. (A) Immunofluorescence photomicrographs of representative spleen sections from WT, TRANCE$^{+/-}$, and TRANCE$^{-/-}$ mice. Spleens were dissected from 3–4-wk-old mice and stained with anti-B220 (FITC) and anti-CD4 (PE). (B) Representative immunofluorescence photomicrographs of spleen sections prepared 10 d after immunization of mice with 50 μg $NP_{12}$-KLH adsorbed to alum. Immunofluorescence microscopy was performed to detect formation of GCs (PNA-biotin/streptavidin–PE and anti-IgM–FITC).

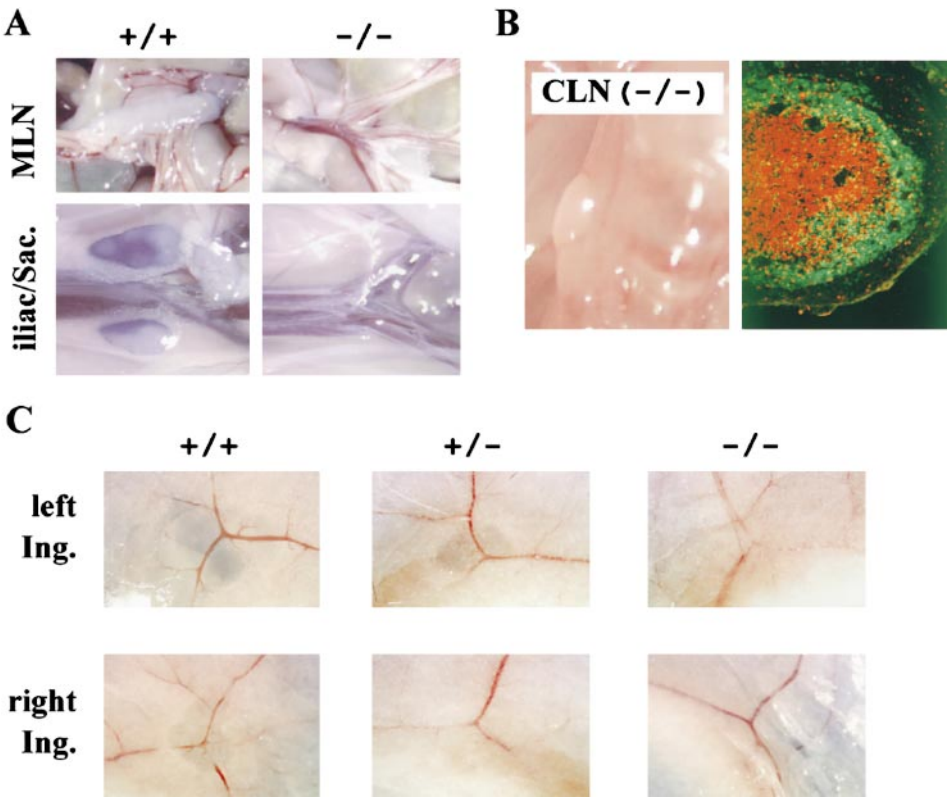



**Figure 3.** TRANCE is essential for proper development of PLNs. (A) Evan's blue dye was injected into all footpads and images of the PLNs were obtained. Shown are MLNs, iliac, and sacral (Sac.) LNs. Apart from the CLNs (see panel B), 100% of TRANCE$^{-/-}$ mice lacked all PLNs and MLNs. (B) The presence of CLNs in TRANCE$^{-/-}$ mice. A representative image of CLNs found in TRANCE$^{-/-}$ mice (left). A representative immunofluorescence analysis of CLNs obtained from TRANCE$^{-/-}$ with anti-CD4–PE and anti-B220–FITC (right). (C) Haploinsufficiency effect of TRANCE on the development of PLNs. A representative image of TRANCE$^{+/-}$ mice lacking one of the inguinal (Ing.) LNs.

*Expression of TRANCE and TRANCE-R in Cells within Developing LNs.* As described above, the most dramatic effect of TRANCE gene deletion is on the development of PLNs and MLNs. As the formation of LN structure is regulated during the early development of mice, we examined TRANCE and TRANCE-R expression in developing LNs. For this study, we isolated MLNs from new-born WT mice (day 0 MLNs).

At the time of birth, MLNs contain various types of hematolymphoid cells. In particular, day 0 MLNs are colonized by CD45$^+$CD4$^+$CD3$^-$ cells, which express surface LT$\alpha\beta$ (Fig. 4), and other gene products (e.g., RelB, IL-2R$\gamma_c$ chain, or BLR-1 [27]), which have been shown to play important roles in LN development (22, 23, 28, 29). We failed to detect surface TRANCE expression in freshly isolated cells from day 0 MLNs by FACS® analysis using TRANCE-R-IgG$_1$ fusion protein (data not shown). However, upon a brief culture of day 0 MLN cells in medium, we could detect surface TRANCE expression specifically on CD45$^+$CD4$^+$CD3$^-$ cells (Fig. 4). The length of culture period between 1 and 2 h did not affect the level of TRANCE expression (data not shown), suggesting that

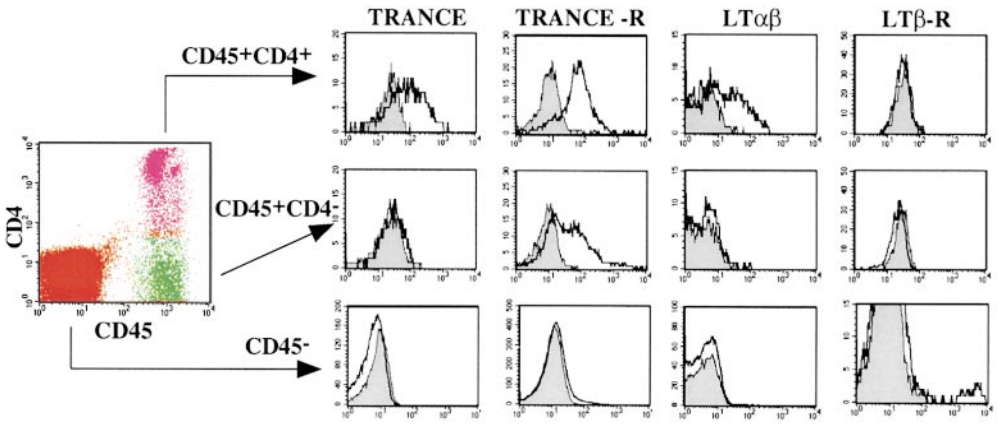



**Figure 4.** CD45$^+$CD4$^+$CD3$^-$ cells in day 0 MLNs express TRANCE and TRANCE-R. Cells obtained from WT day 0 MLNs were stained with anti-CD4, anti-CD45, and TRANCE-R-IgG$_1$ (for TRANCE), 1E6.66 (for anti–TRANCE-R), LT-$\beta$R-IgG$_1$ (for surface LT$\alpha\beta$), or AF.H6 (for anti–LT-$\beta$R) (thick lines). Cells were isolated by mechanical disruption of day 0 MLNs. Similar results were also obtained when cells were isolated by collagenase digestion of day 0 MLNs (data not shown). For detection of surface TRANCE, cells obtained from day 0 MLNs were cultured in medium for 1–2 h before staining with TRANCE-R-IgG1. Gray histograms are negative controls. For TRANCE-R-IgG$_1$ and LT-$\beta$R-IgG$_1$, incubation with human IgG$_1$ was performed as a negative control. For anti–TRANCE-R mAb (1E6.6) and anti–LT-$\beta$R Ab (AF.H6), rat IgG and hamster anti-KLH (Ha/48) were used as negative controls, respectively.

surface TRANCE might have been shed due to its continuous interaction with the receptor in developing MLNs. The restricted expression of TRANCE on $CD45^+CD4^+CD3^-$ cells was similar to that of surface LTαβ (Fig. 4).

We have also examined the expression of surface TRANCE-R by FACS® analysis using anti–TRANCE-R mAb (1E6.66) on cells from day 0 MLNs. Unlike TRANCE, TRANCE-R was readily detected in freshly isolated $CD45^+CD4^+CD3^-$ cells as well as in some $CD45^+CD4^-$ cells (Fig. 4). TRANCE-$R^+CD45^+CD4^-$ cells were also shown to express B220, CD19, and surface IgM, indicating that they are B cells (data not shown). However, TRANCE-R was not detected on $CD45^-$ cells. In contrast to the expression of TRANCE-R on cells of hematolymphoid lineages, LT-βR expression was detected only on a subpopulation of $CD45^-$ stromal or endothelial cells found in day 0 MLNs (Fig. 4).

Thus, these results indicate that, although both TRANCE and LTαβ are expressed on the same population of cells (i.e., $CD45^+CD4^+CD3^-$) colonizing the developing LNs, TRANCE-R and LT-βR are expressed on a discrete population of cells, suggesting that TRANCE and LTαβ may affect different cell types within developing LNs.

The colonization of developing LNs by $CD45^+CD4^+CD3^-$ cells has been of particular interest, as these cells express surface LTαβ and other gene products that are essential for LN genesis (27). As TRANCE, similar to LTαβ, is also expressed specifically on $CD45^+CD4^+CD3^-$ cells in developing LNs (day 0 MLNs), we speculated that the fate of those cells in developing LNs might be regulated by TRANCE or LTαβ during LN development. To test this hypothesis, we have collected day 0 MLNs from WT mice and rMLNs, which is the area where MLNs should have developed, from newborn TRANCE$^{-/-}$ and LTα$^{-/-}$ mice (Fig. 5 A). Day 0 rMLNs from TRANCE$^{-/-}$ (Fig. 5 A) and LTα$^{-/-}$ mice (data not shown) were connected to the lymphatic vessels from the small intestines and filled with milky white fluid (probably chyle) at the time of killing, indicating that TRANCE$^{-/-}$ mice have developed lymphatic vessels (stage II), as do LTα$^{-/-}$ mice.

In WT day 0 MLNs, ~50% of $CD45^+$ cells are $CD4^+CD3^-$ cells, all of which express $\alpha_4\beta_7$ integrin (measured by FACS® analysis using anti-$\alpha_4\beta_7$ mAb, LPAM-1; Fig. 5, B and C), which is the ligand for MAdCAM-1, a dominant addressin on high endothelial venules in developing MLNs (26, 27, 30). When the topology of these $CD45^+CD4^+$

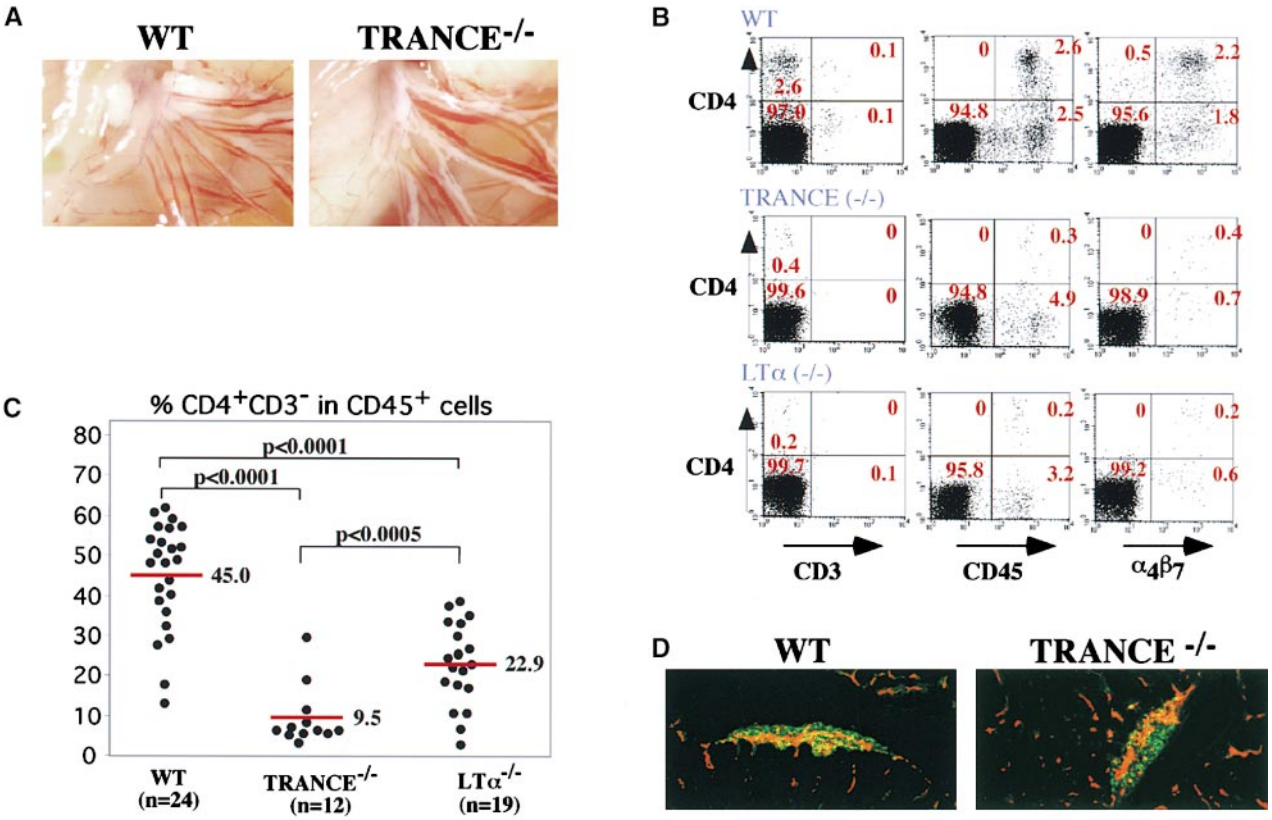



**Figure 5.** Reduction of colonization by $CD45^+CD4^+CD3^-$ cells and their failure to form clusters in TRANCE$^{-/-}$ or LTα$^{-/-}$ mice. (A) A representative image of MLNs from newborn WT mice and of rMLNs found in newborn TRANCE$^{-/-}$ mice. Dotted circles indicate MLNs or rMLNs isolated. Similar rMLNs were observed in newborn LTα$^{-/-}$ mice (data not shown). Lymphatic vessels are white, presumably due to the chyle being drained from intestines. (B) A representative flow cytometric analysis of cells taken from day 0 MLNs from WT mice or rMLNs from TRANCE$^{-/-}$ and LTα$^{-/-}$ mice. (C) The percentage of $CD45^+$ cells showing the $CD4^+CD3^-$ phenotype in day 0 MLNs from WT mice or rMLNs from TRANCE$^{-/-}$ and LTα$^{-/-}$ mice. (D) Representative immunofluorescence analysis of frozen sections obtained from day 0 intestines from WT or TRANCE$^{-/-}$ mice. Sections were stained with anti-CD4–Alexa-488 and anti–MAdCAM-1–PE.

$CD3^-$ cells in the developing LNs was examined by staining the freshly isolated whole day 0 MLNs, we found that these cells were organized into compact clusters in discrete regions of day 0 MLNs (Fig. 6). Moreover, $CD45^+CD4^+CD3^-$ cells were identified as clusters colocalizing with MAdCAM-$1^+$ endothelial venules (Fig. 6).

In day 0 rMLNs of TRANCE$^{-/-}$ mice, the overall percentage of $CD45^+$ cells was significantly reduced (about a fourfold reduction) compared with that in day 0 MLNs of WT mice (data not shown). In addition to the overall reduction of $CD45^+$ cells, there was a selective decrease in the percentage of $CD45^+CD4^+CD3^-$ cells in day 0 rMLNs from TRANCE$^{-/-}$ mice (Fig. 5, B and C) such that only ~10% of $CD45^+$ cells were $CD45^+CD4^+CD3^-$ cells. Although reduced in numbers, the $CD45^+CD4^+CD3^-$ cells found in day 0 rMLNs from TRANCE$^{-/-}$ mice still expressed $\alpha_4\beta_7$ integrin at levels comparable to those of WT cells (Fig. 5, B and C). In addition to the reduced number of $CD45^+CD4^+CD3^-$ cells in day 0 rMLNs observed by FACS® analysis, immunofluorescence analysis showed that the few $CD45^+CD4^+CD3^-$ cells that were present were found scattered over the entire rMLNs, in contrast to cluster-forming $CD45^+CD4^+CD3^-$ cells in WT day 0 MLNs (Fig. 6). In addition, the network of MAdCAM-$1^+$ endothelial venules, which were observed in day 0 MLNs from WT mice, was dramatically underdeveloped in TRANCE$^{-/-}$ mice (Fig. 6).

For comparison, we also examined rMLNs from newborn LT$\alpha^{-/-}$ mice, which also fail to develop LNs. Similar to TRANCE$^{-/-}$ mice, LT$\alpha^{-/-}$ mice showed a reduction in the percentage of $CD45^+$ cells in day 0 rMLNs (data not shown). In addition, a significant reduction (about twofold) in the percentage of $CD4^+CD3^-$ cells within the $CD45^+$ population was observed in day 0 rMLNs of LT$\alpha^{-/-}$ mice (Fig. 5, B and C). The remaining cells expressed normal levels of $\alpha_4\beta_7$ integrin (Fig. 5, B and C). Compared with day 0 rMLNs from TRANCE$^{-/-}$ mice, more $CD45^+CD4^+CD3^-$ cells were observed in LT$\alpha^{-/-}$ day 0 rMLNs (Fig. 5, B and C). However, as in TRANCE$^{-/-}$ mice, the $CD45^+CD4^+CD3^-$ cells were scattered throughout the rMLNs (Fig. 6). The network of MAdCAM-$1^+$ endothelial venules as well as their thickness was also dramatically diminished compared with day 0 MLNs from WT mice (Fig. 6).

In contrast to developing LNs, day 0 intestines from WT mice and TRANCE$^{-/-}$ mice contained a comparable percentage of $CD4^+CD3^-$ cells within the $CD45^+$ population (data not shown), which are identified as clusters (Fig. 5 D). Day 0 intestines from LT$\alpha^{-/-}$ mice were previously shown to contain $CD4^+CD3^-$ cells, but failed to form clusters (31).

Taken together, these results indicate that the TNF family members TRANCE and LT$\alpha\beta$ regulate the colonization of developing LNs by hematolymphoid lineage cells, in particular by $CD45^+CD4^+CD3^-$ cells, either by affecting their migration into the developing LNs or by affecting their persistence/differentiation within the LN anlage. Moreover, the ability of $CD45^+CD4^+CD3^-$ cells to form clusters in developing LNs is also regulated by TRANCE and LT$\alpha\beta$.

*Ectopic Expression of TRANCE Transgene Restores LNs and $CD45^+CD4^+CD3^-$ Cells to TRANCE$^{-/-}$ Mice.* To determine whether ectopic expression of TRANCE can restore LN development in TRANCE$^{-/-}$ mice, we crossed TRANCE$^{-/-}$ mice to TRANCE.TG-1, a transgenic mouse line overexpressing TRANCE in T and B cells (see Materials and Methods). As reported previously, FACS® analysis of freshly isolated resting mature T or B cells using TRANCE-R-$IgG_1$ fusion protein does not reveal any significant surface TRANCE expression in WT mice (25). However, transgenic (or overexpressed) TRANCE was readily detected on most of the freshly isolated mature T or B cells from TRANCE.TG-1 mice (data not shown).

In newborn TRANCE.TG-1 mice, transgenic TRANCE was overexpressed in $CD4^+CD8^+$ thymocytes (Fig. 7 A). When freshly isolated day 0 MLN cells from TRANCE. TG-1 mice were analyzed, a large number of $B220^+$

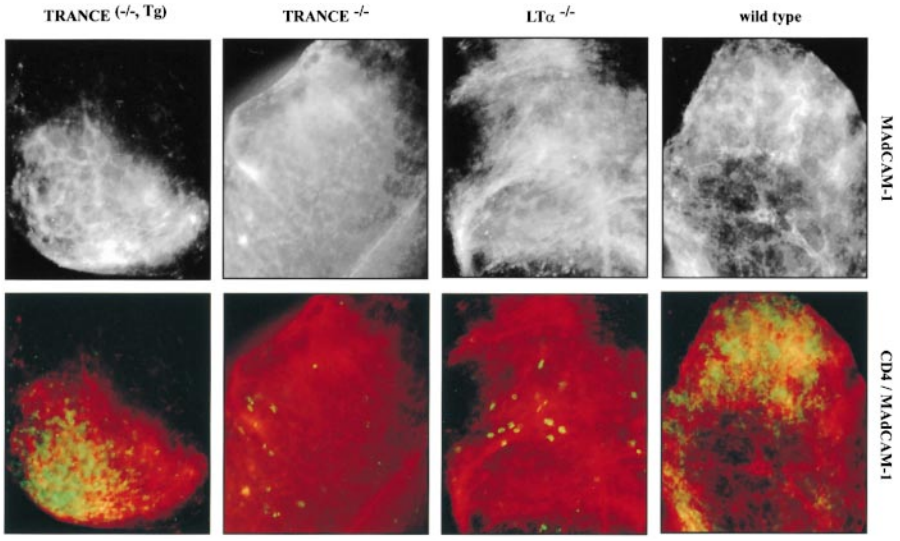
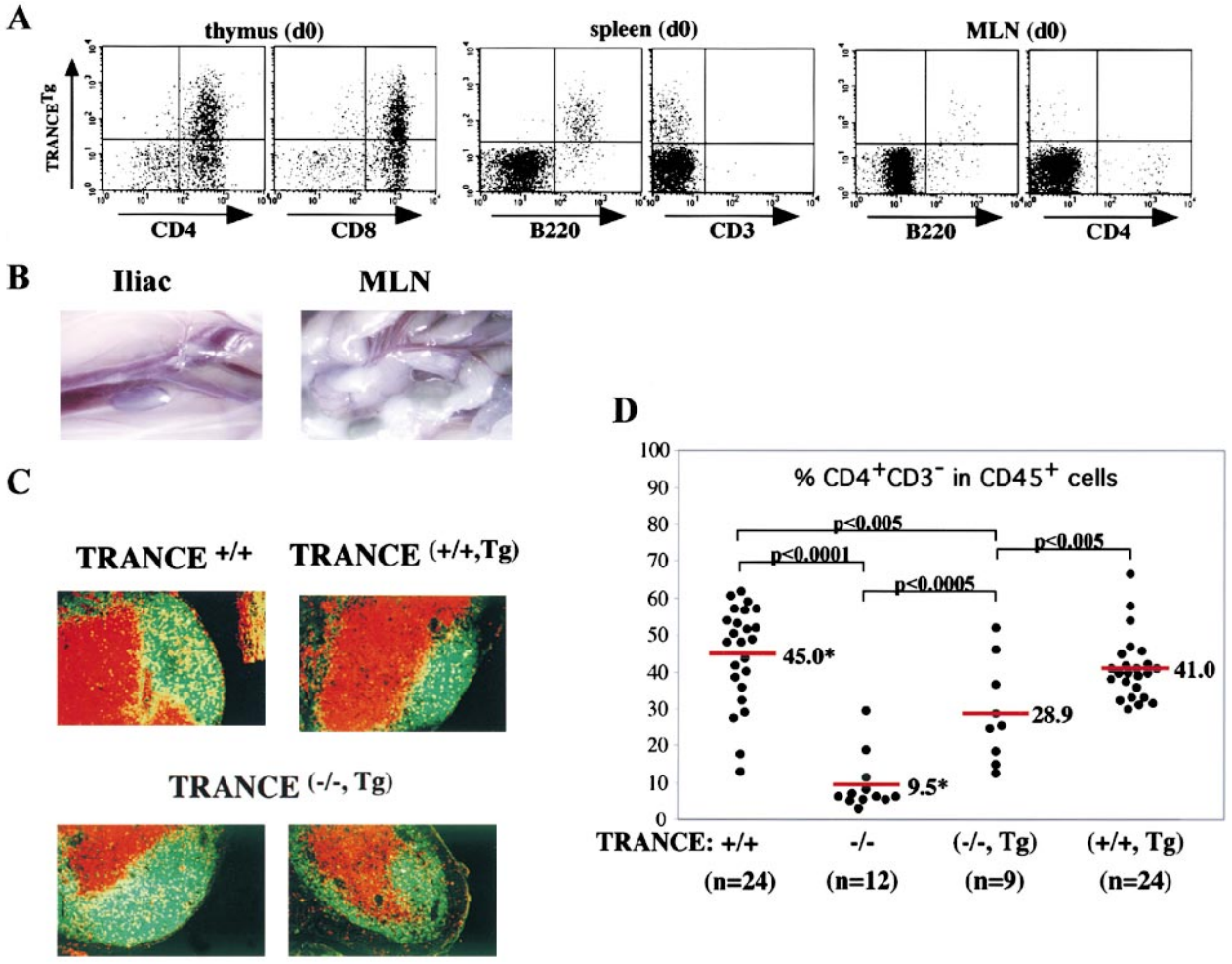



**Figure 6.** Both TRANCE and LT-α are required for cluster formation by $CD45^+CD4^+CD3^-$ cells and for the formation of MAdCAM-$1^+$ endothelial cell network within developing LNs. Representative images taken from whole mount staining of freshly isolated MLNs from newborn WT or TRANCE$^{(-/-,Tg)}$ mice, and rMLNs from TRANCE$^{-/-}$ or LT$\alpha^{-/-}$ mice. The whole MLNs or rMLNs were stained with anti-CD4–FITC and anti–MAdCAM-1–PE.

$CD19^+$ cells overexpressing TRANCE, but not $CD3^+$ cells, were observed (Fig. 7 A). Furthermore, we failed to detect overexpressed TRANCE on freshly isolated $CD45^+$ $CD4^+CD3^-$ cells in day 0 MLNs from TRANCE. TG-1 mice. Although overexpressed TRANCE in day 0 MLNs was detected only in B cells, the cell lineage delivering the TRANCE-dependent signal for LN development in the transgenic mice is not known.

Although transgenic TRANCE was not detected on $CD45^+CD4^+CD3^-$ cells in developing MLNs, there was a significant restoration of all PLNs when adult $TRANCE^{-/-}$ mice carrying the TRANCE transgene, $TRANCE^{(-/-,Tg)}$,




E

| Percentage of Lymph Nodes restored in $TRANCE^{(-/-,Tg)}$ mice | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Lymph Nodes | Mand. | Super. | Cerv. | Axi. | Brach. | Mesen. | Ingui. | Lumb. | Iliac | Sac | Pop. |
| Percentage | 70.6 | 11.8 | 94.1 | 11.8 | 35.3 | 100* | 41.2 | 29.4 | 70.6 | 52.9 | 5.9 |

**Figure 7.** Ectopic expression of TRANCE restores LN development in $TRANCE^{-/-}$ mice. (A) Transgenic TRANCE is overexpressed in T and B cells, but not in $CD4^+CD3^-$ cells found in day 0 MLNs. Freshly isolated cells were incubated with the indicated Abs and examined by FACS® analysis. (B) Representative images of PLNs (Iliac) and MLNs observed in $TRANCE^{(-/-,Tg)}$ mice. 4 h after injection of Evan's blue dye, LNs were imaged. Note that one of the two iliac LNs and sacral LNs were still missing in the $TRANCE^{(-/-,Tg)}$ mouse shown here. In addition, MLNs in these mice were smaller than those in WT mice. (C) Representative immunofluorescence analysis of LNs from WT, $TRANCE^{(+/+,Tg)}$, and $TRANCE^{(-/-,Tg)}$ mice. Sections were stained with anti-CD4–PE and anti-B220–FITC. Note that the righthand image in $TRANCE^{(-/-,Tg)}$ shows disrupted primary B cell follicle. (D) The percentage of $CD45^+$ cells showing the $CD4^+CD3^-$ phenotype in day 0 MLNs from $TRANCE^{(+/+,Tg)}$ and $TRANCE^{(-/-,Tg)}$ mice. *For comparison, the percentages of $CD45^+$ cells showing the $CD4^+CD3^-$ phenotype in day 0 MLNs from WT or rMLNs from $TRANCE^{-/-}$ mice are included, which were taken from Fig. 5 C. (E) The percentage of LNs restored in $TRANCE^{(-/-,Tg)}$ mice. *Although all of the $TRANCE^{(-/-,Tg)}$ mice contained MLNs, the number of MLNs averaged two, compared with four to six MLNs found in WT mice. Mand., mandibular; Super., superficial; Cerv., cervical; Axi., axial; Brach., brachial; Mesen., mesentery; Ingui., inguinal; Lumb., lumbar; Sac, sacral; Pop., popliteal.

were analyzed (Fig. 7, B and E). In all cases, TRANCE$^{(-/-,Tg)}$ mice developed MLNs, though they were smaller than MLNs in WT mice. Moreover, immunofluorescence analysis of these restored LNs revealed that they retained almost normal cellular architecture, although the formation of B cell follicles was somewhat defective (Fig. 7 C).

As we have shown above that day 0 rMLNs from TRANCE$^{-/-}$ mice contained reduced numbers of CD45$^+$ cells and were not colonized by cluster-forming CD45$^+$CD4$^+$CD3$^-$ cells, we also examined whether ectopic expression of TRANCE affected the fate of hematolymphoid lineage cells in the developing MLNs of TRANCE$^{(-/-,Tg)}$ mice. Although TRANCE$^{(-/-,Tg)}$ mice developed MLNs, day 0 MLNs from these mice were still significantly smaller and showed lower representation of CD45$^+$ cells than those of WT or TRANCE.TG-1 littermates such that the percentage of CD45$^+$ cells from day 0 MLN of TRANCE$^{(-/-,Tg)}$ mice was statistically not different from that of TRANCE$^{-/-}$ mice (data not shown). However, day 0 MLNs of TRANCE$^{(-/-,Tg)}$ mice contained a significantly higher percentage of CD4$^+$CD3$^-$ cells within the CD45$^+$ population than day 0 rMLNs of TRANCE$^{-/-}$ mice (Fig. 7 D). Despite the increased colonization, the percentage of CD4$^+$CD3$^-$ cells within the CD45$^+$ population in day 0 MLNs from TRANCE$^{(-/-,Tg)}$ mice was still significantly lower than that from WT or TRANCE.TG-1 littermates (Fig. 7 D), and in fact similar to that observed in day 0 rMLNs from LT$\alpha^{-/-}$ mice (Figs. 5 C and 7 D). However, transgenic TRANCE expression restored the cluster formation of CD45$^+$CD4$^+$CD3$^-$ cells such that most of the CD45$^+$CD4$^+$CD3$^-$ cells were identified as clusters in discrete regions of day 0 MLNs (Fig. 6). Moreover, the colocalization of MAdCAM-1$^+$ endothelial venules with clusters of CD4$^+$CD3$^-$ cells could be observed in day 0 MLNs from TRANCE$^{(-/-,Tg)}$ mice (Fig. 6).

As transgenic expression of TRANCE could partially rescue the reduced numbers of CD45$^+$CD4$^+$CD3$^-$ cells and also the lack of LNs in TRANCE$^{-/-}$ mice, we addressed whether overexpression of TRANCE in LT$\alpha^{-/-}$ mice could restore the defects in these mice. However, generation of neither LNs nor PP formation could be restored by overexpression of TRANCE in LT$\alpha^{-/-}$ mice (data not shown).

Similar to TRANCE.TG-1 mice, TRANCE.TG-3 mice, which express transgenic TRANCE only on T cells, also rescued LN development in TRANCE$^{-/-}$ mice, though at different degrees (data not shown). Thus, these results allow us to make the following conclusion: restricted restoration of TRANCE expression in TRANCE$^{-/-}$ mice was sufficient to support LN genesis and also increased the relative number of CD4$^+$CD3$^-$ cells in the CD45$^+$ population, their cluster formation, and the formation of MAdCAM-1$^+$ endothelial venules.

## Discussion

TRANCE is a member of the TNF family, which has been shown to play diverse roles in maintaining proper bone homeostasis and also in regulating the immune system in adult animals (32). Inactivation of the murine TRANCE gene revealed its pivotal role during the development of LNs (this work and reference 17). The role of the TRANCE/TRANCE-R system during LN organogenesis parallels that of the LT/LT-βR system, which is also essential for LN development (6). In contrast, although the TRANCE/TRANCE-R system is essential for LN development, it is dispensable for PP development, which requires the LT/LT-βR system.

Over the past several years, a number of additional gene products were shown to play critical roles during the development of LNs. For example, in addition to the TRANCE/TRANCE-R or LTαβ/LT-βR systems, various transcription regulators such as Id2 and RelB, signal transducers TRAF6 and NIK, and a chemokine receptor, BLR-1, were shown to be important for LN genesis (7–10, 13, 14, 19, 20, 22, 23, 27–29, 33, 34). However, how and which steps of LN development are regulated by these factors are not well understood.

Nonetheless, recent experiments have suggested that colonization of developing LNs or PPs by $\alpha_4\beta_7^+$CD4$^+$CD3$^-$ cells might be critical for the subsequent maturation of these secondary lymphoid organs (27, 33). This hypothesis was based on the observation that developing LNs or PPs collect the $\alpha_4\beta_7^+$CD4$^+$CD3$^-$ cells, which express LTαβ, RelB, IL-7Rα, IL-2R$\gamma_c$, and BLR-1, all of which were shown to be important for the genesis of LNs or PPs (7, 8, 13, 14, 22, 23, 27–29, 33, 34).

The fate of CD45$^+$CD4$^+$CD3$^-$ cells during the early development of LNs from various LN-less mutant mice has not been examined to date. However, some progress has been made in analyzing those cells in developing PPs. For example, no CD4$^+$CD3$^-$IL-7Rα$^+$ cells were detected in the fetal intestines of Id2$^{-/-}$ mice, which failed to develop PPs (35). In addition, when developing intestines from IL-7Rα, Janus kinase 3, or IL-2R$\gamma_c$ knockout mice that lack PPs were examined, no discrete clusters of CD4$^+$CD3$^-$ cells were detected (31, 33, 34, 36). The lack of clusters of CD4$^+$CD3$^-$ cells in developing intestines was also observed in LT$\alpha^{-/-}$ mice, which failed to develop PPs (31). Taken together, these results suggested that the presence of clusters of $\alpha_4\beta_7^+$CD4$^+$CD3$^-$ correlates with the development of PPs.

In this study, we have examined the fate of $\alpha_4\beta_7^+$CD4$^+$CD3$^-$ cells in developing LNs and also examined how TRANCE or LTαβ might affect them. We have first shown that both TRANCE and TRANCE-R are expressed on $\alpha_4\beta_7^+$CD4$^+$CD3$^-$ cells within developing LNs. Although B cells found in early MLNs also express TRANCE-R, it is most likely that TRANCE regulates the fate of TRANCE-R$^+$CD4$^+$CD3$^-$ cells in an autocrine or paracrine manner, as B cells are not required for LN formation. In addition, similar to TRANCE, LTαβ expression is restricted to $\alpha_4\beta_7^+$CD4$^+$CD3$^-$ cells, whereas LT-βR is expressed only on a subpopulation of CD45$^-$ cells, indicating that the initial target cells for TRANCE and LT are distinct.

When rMLNs from newborn TRANCE$^{-/-}$ mice were analyzed, there was a significant reduction in the percentage of CD45$^+$CD4$^+$CD3$^-$ cells among leukocytes. Thus, these results indicated that TRANCE indeed regulates the degree of colonization by CD45$^+$CD4$^+$CD3$^-$ cells within developing LNs. Although reduced in numbers, CD45$^+$CD4$^+$CD3$^-$ cells found in day 0 rMLNs from TRANCE$^{-/-}$ mice expressed normal levels of $\alpha_4\beta_7$ integrin, which is the ligand for MAdCAM-1, a dominant addressin found in the high endothelial venules of developing MLNs. This suggests that in the absence of TRANCE, CD45$^+$CD4$^+$CD3$^-$ cells can still differentiate from their precursors. Consistent with this notion, normal colonization by CD45$^+$CD4$^+$CD3$^-$ cells was observed in developing intestines from TRANCE$^{-/-}$ mice. In addition, TRANCE$^{-/-}$ mice have normal development of NK cells, which can also be found as progeny from CD45$^+$ CD4$^+$CD3$^-$ precursor cells (27). This apparently normal development of CD4$^+$CD3$^-$ cells in the fetal intestines makes the defects of TRANCE$^{-/-}$ mice distinct from those of Id2$^{-/-}$ mice, which showed a total lack of CD4$^+$CD3$^-$ precursor cells and NK cells (35). Therefore, it is likely that TRANCE is required for maintenance of colonization by CD45$^+$CD4$^+$CD3$^-$ cells specifically within the developing LNs in situ.

The reduction in the number of CD45$^+$CD4$^+$CD3$^-$ cells in developing LNs but not in PPs suggests that distinct factors other than TRANCE are produced in fetal intestines to support the continued colonization by CD45$^+$CD4$^+$ CD3$^-$ cells. Conversely, it is possible that the few CD45$^+$ CD4$^+$CD3$^-$ cells found in day 0 rMLNs from TRANCE$^{-/-}$ mice could be the result of the limited amounts of these intestinal factors present in developing LNs.

In addition to the failure of colonization, CD45$^+$CD4$^+$ CD3$^-$ cells found in rMLNs from newborn TRANCE$^{-/-}$ mice failed to organize into discrete clusters. Similar to TRANCE$^{-/-}$ mice, LT$\alpha^{-/-}$ mice also had defects in colonization and cluster formation by CD45$^+$CD4$^+$CD3$^-$ cells in day 0 rMLNs. Thus, the cluster formation of CD45$^+$ CD4$^+$CD3$^-$ cells as well as their colonization within developing LN anlage seems to be critical for proper LN development. Indeed, when day 0 MLNs from TRANCE$^{(-/-,Tg)}$ mice were examined, we showed that the degree of colonization by CD45$^+$CD4$^+$CD3$^-$ cells, though increased compared with TRANCE$^{-/-}$ mice, was still low and even comparable to that found in LT$\alpha^{-/-}$ mice. However, in contrast to LT$\alpha^{-/-}$ mice, CD45$^+$CD4$^+$CD3$^-$ cells were organized into discrete clusters in day 0 MLNs of TRANCE$^{(-/-,Tg)}$ mice.

Taken together, our studies establish that two TNF family systems, TRANCE/TRANCE-R and LT$\alpha\beta$/LT-$\beta$R, regulate the colonization and cluster formation by CD45$^+$ CD4$^+$CD3$^-$ cells within developing LNs, potentially in sequential events, and suggest that both events are required for proper LN development. In addition, we showed that in the absence of either system, the network of MAdCAM-1$^+$ endothelial venules is not fully established, which results in the arrest of LN development at the stage where LN anlage needs to be expanded with various leukocytes. Thus, we speculate that the TRANCE/TRANCE-R or LT/LT-$\beta$R system is required for LN anlage to continue stage III development, but not for the initiation of colonization by hematolymphoid cells within developing LN anlage, which begins stage III (see Introduction). Moreover, our results now suggest that the mode of regulation of LN development by CD45$^+$CD4$^+$CD3$^-$ cells appear to parallel that of PP development, such that these cells also colonize and form clusters in developing intestines (31).

So how might TRANCE or LT$\alpha\beta$ regulate the fate of CD45$^+$CD4$^+$CD3$^-$ cells within developing LNs, which in turn are critical for subsequent LN genesis? In the case of TRANCE, one can speculate the following scenarios. First, TRANCE may work as a survival factor for CD45$^+$ CD4$^+$CD3$^-$ cells. This is intriguing, as it was previously shown that TRANCE enhances the viability of dendritic cells, for which CD45$^+$CD4$^+$CD3$^-$ cells can be precursor cells (27, 37). However, transgenic overexpression of Bcl-2 in all CD45$^+$ cells failed to restore the LN development in TRANCE$^{-/-}$ mice (data not shown), suggesting that a simple enhancement of the viability of those CD45$^+$ CD4$^+$CD3$^-$ cells by TRANCE might not be the critical mechanism. Another possibility would be that TRANCE regulates the activation/differentiation of CD45$^+$CD4$^+$ CD3$^-$ cells within developing LNs in situ. This is a likely possibility, as TRANCE has been shown to be a differentiation factor during osteoclast development (38, 39), and also to induce various cytokines and chemokines in dendritic cells or osteoclasts (our unpublished data). Finally, the TRANCE/TRANCE-R system might regulate the migration of CD45$^+$CD4$^+$CD3$^-$ cells into developing LN anlage. Although this possibility is consistent with the reduced number of CD45$^+$CD4$^+$CD3$^-$ cells and the reduced network of MAdCAM-1$^+$ endothelial venules observed in day 0 rMLNs from mutant mice, the fact that these cells and structures exist in the nascent LNs suggests that TRANCE is not essential for the initiation of migration.

Although the deficiency of TRANCE or LT$\alpha$ arrests LN development at a similar stage and alters the fate of CD45$^+$CD4$^+$CD3$^-$ cells similarly in LN anlage, it appears that the LT$\alpha\beta$/LT-$\beta$R systems regulates LN genesis independently of the TRANCE/TRANCE-R system. In support, we showed in this study that the initial target cells of TRANCE or LT$\alpha\beta$ ligands are distinct, as TRANCE-R and LT-$\beta$R are expressed on CD45$^+$CD4$^+$CD3$^-$ cells and CD45$^-$ stromal cells, respectively. This also suggests that the fate of CD45$^+$CD4$^+$CD3$^-$ cells must be regulated indirectly by LT$\alpha\beta$. Second, TRANCE-TG.1, which restored LN development in TRANCE$^{-/-}$ mice, failed to rescue the defects in LN organogenesis of LT$\alpha^{-/-}$ mice. In addition, administration of agonistic anti–LT-$\beta$R Ab into developing TRANCE$^{-/-}$ embryos failed to restore LN development despite normal LT-$\beta$R expression in day 0 rMLNs from these mice (data not shown), whereas it did in LT$\alpha^{-/-}$ mice as reported previously (13).

However, the similar defects in the fate of CD45$^+$ CD4$^+$CD3$^-$ cells observed within rMLNs from TRANCE-

or LTα-deficient mice suggest that TRANCE/TRANCE-R and LTαβ/LT-βR systems must work in a similar fashion. Considering the fact that the number of $CD45^+CD4^+CD3^-$ cells found in day 0 rMLNs is always lower in the $TRANCE^{-/-}$ mice than in $LT\alpha^{-/-}$, and that $LT\alpha^{-/-}$ day 0 rMLNs contained a comparable number of $CD45^+CD4^+CD3^-$ cells compared with $TRANCE^{(-/-,Tg)}$, which developed LNs, we postulate the following regulation of LN development by TRANCE and LTαβ ligands. The TRANCE/TRANCE-R system operates at the beginning of stage III when $\alpha_4\beta_7^+$ $CD4^+CD3^-$ cells start migrating into LN anlage and supports the continued colonization by $CD45^+CD4^+CD3^-$ cells within the developing LN anlage. When the number of $CD45^+$ $CD4^+CD3^-$ cells reaches a critical point, the interaction between $CD45^+CD4^+CD3^-$ cells and stromal cells via the LTαβ/LT-βR system leads to the homotypic interaction of $CD45^+CD4^+CD3^-$ cells to form compact clusters. These clusters of $CD45^+CD4^+CD3^-$ cells provide a community effect for subsequent differentiation of $CD45^+$ $CD4^+CD3^-$ cells themselves and other surrounding cells (i.e., MAdCAM-$1^+$ endothelial cells) within developing LN anlage. Such a model is similar to the community effect that has been described for tissue formation arising from a variety of different types of precursor cells (40).

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