LTβR Signaling Induces Cytokine Expression and Up-Regulates Lymphangiogenic Factors in Lymph Node Anlagen[1]

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The formation of lymph nodes is a complex process crucially controlled through triggering of LTβR on mesenchymal cells by $LT\alpha_1\beta_2$ expressing lymphoid tissue inducer (LTi) cells. This leads to the induction of chemokines to attract more hematopoietic cells and adhesion molecules to retain them. In this study, we show that the extravasation of the first hematopoietic cells at future lymph node locations occurs independently of LTα and that these cells, expressing TNF-related activation-induced cytokine (TRANCE), are the earliest LTi cells. By paracrine signaling the first expression of $LT\alpha_1\beta_2$ is induced. Subsequent LTβR triggering on mesenchymal cells leads to their differentiation to stromal organizers, which now also start to express TRANCE, IL-7, as well as VEGF-C, in addition to the induced adhesion molecules and chemokines. Both TRANCE and IL-7 will further induce the expression of $LT\alpha_1\beta_2$ on newly arrived immature LTi cells, resulting in more LTβR triggering, generating a positive feedback loop. Thus, LTβR triggering by LTi cells during lymph node development creates a local environment to which hematopoietic precursors are attracted and where they locally differentiate into fully mature, $LT\alpha_1\beta_2$ expressing, LTi cells. Furthermore, the same signals may regulate lymphangiogenesis to the lymph node through induction of VEGF-C. ***The Journal of Immunology,*** **2009, 182: 5439–5445.**

The development of lymph nodes (LNs)[3] depends on the crosstalk between hematopoietic cells and mesenchymal stromal cells (1–6), whereby the inductive signal for lymph node development comes from $CD4^+IL\text{-}7R^+CD3^-CD45^+ROR\gamma t^+$ hematopoietic cells. These lymphoid tissue inducer (LTi) cells express lymphotoxin-$\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) and are thought to trigger lymphotoxin β receptor (LTβR) expressed on stromal cells (2, 7, 8). The importance of the LTβR signaling lies in the induction of adhesion molecules and production of chemokines that start a set of events leading to the formation of the lymph node. Initially, LTβR ligation leads to activation of the classical NF-κB pathway, which results in the expression of proinflammatory molecules such as the chemokines MIP1β, MIP-2, and the adhesion molecule VCAM-1. Prolonged triggering results in the activation of the alternative NF-κB pathway and the generation of p52, involved in the transcription of the lymphoid chemokines such as CCL21, CCL19, and CXCL13 (9, 10). These chemokines in particular are crucial for the development of the lymph node (2, 11–15).

The expression of $LT\alpha_1\beta_2$ itself can equally well be induced by signaling through the IL-7R as well as the TNF-related activation-induced cytokine-receptor (TRANCE-R; also known as Rank, Tnfrsf11a, ODFR, or Ly109) upon binding of the respective ligands, IL-7 or TRANCE (3). For lymph node development, it was shown that signaling through both receptors is critical, while during Peyer's patch formation only IL-7R triggering is mandatory (2, 3, 5, 15–17). Although TRANCE has been reported to be expressed by LTi as well as stromal organizer cells in developing lymph nodes, IL-7 was shown to be expressed in stromal organizer cells of developing Peyer's patches (8, 18, 19). The importance of limited IL-7R triggering for lymphoid organ development can be deduced from experiments in which transgenic overexpression of IL-7 resulted in an increased number of LTi cells, leading to a 5-fold increase in the number of Peyer's patches and the formation of ectopic lymphoid structures (20). In contrast, transgenic overexpression of TRANCE did not affect the number of LTi cells on a wild-type (WT) background, while it was able to rescue the reduced number of LTi cells in developing lymph nodes of $TRANCE^{-/-}$ mice (18). It is unclear how expression of TRANCE and IL-7 is regulated during lymph node development, although analysis of the TRANCE promoter region revealed response elements for vitamin D_3 and glucocorticoids (21, 22), as well as binding sites for Runx2 and NF-κB (23), while LTβR signaling has been proposed to result in enhanced IL-7 production (20). However, it has been reported that initial clustering of LTi cells can occur in $LT\alpha^{-/-}$ mice, and therefore other mechanisms precede the crucial triggering of LTβR on stromal organizer cells (24, 25).

In this study, we show that the expression of TRANCE on LTi cells is unaffected in LN anlagen from $LT\alpha^{-/-}$ mice, but that $LT\alpha_1\beta_2$-mediated LTβR signaling is essential for the expression of TRANCE on stromal cells. The earliest phase of LN development is marked by the clustering of LTi cells, which occurs

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[3] Abbreviations used in this paper: LN, lymph node; LTi, lymphoid tissue inducer; $LT\alpha_1\beta_2$, lymphotoxin-$\alpha_1\beta_2$; LTβR, lymphotoxin β receptor; WT, wild type; MAdCAM-1, anti-mucosal addressin cell adhesion molecule-1; LTα, lymphotoxin α; MEF, mouse embryonic fibroblasts; rMLN, rudimentary mesenteric LN; ALN, axillary LN; BLN, brachial LN; LEC, lymphatic endothelial cell; TRANCE, TNF-related activation-induced cytokine.

independent of $LT\alpha_1\beta_2$-LTβR, followed by the $LT\alpha_1\beta_2$-mediated differentiation of stromal cells. The $LT\alpha_1\beta_2$-induced differentiation of stromal cells leads to the up-regulation of VCAM-1, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), and ICAM-1, as well as to the induction of TRANCE and IL-7 expression, which will contribute to enhanced expression of $LT\alpha_1\beta_2$ on LTi cells. Furthermore, LTβR triggering on mesenchymal cells by LTi cells results in the production of VEGF-C, which points to an additional role of LTi cells in the regulation of lymphangiogenesis to the developing lymph nodes.

Materials and Methods

Animals

C57BL/6 mice were purchased from Harlan Sprague Dawley, lymphotoxin α $(LT\alpha)^{-/-}$ mice on the C57BL/6 background were purchased from Charles River Laboratories. Both mouse strains were bred in our in house facilities and kept under routine laboratory conditions. The Animal Experiments Committee of the VU (Vrije Universiteit) University Medical Center approved all of the experiments described in this study.

Timed pregnancies

Mice were mated overnight, and the day of vaginal-plug detection was marked as E0.5. Pregnant females were sacrificed at different time points, and embryos were harvested and stored in OCT embedding medium (Sakura Finetek Europe).

Immunofluorescence

Seven-μm cryosections were fixed in dehydrated acetone for 2 min and air-dried for an additional 15 min. Endogenous avidin was blocked with an avidin-biotin block (Vector Laboratories). Sections were then preincubated in PBS supplemented with 5% (v/v) mouse serum for 10 min. Incubation with primary Ab for 45 min was followed by a 30 min incubation with Fluor-Alexa-labeled conjugate (Invitrogen Life Technologies) when needed. All incubations were conducted at room temperature. Sections were counter stained with Hoechst 33342 (Invitrogen Life Technologies) for 10 min. For detection of LN anlagen, visible as clusters of LTi cells and their precursors, serial sections of embryos were collected and every twentieth section was stained for CD4 (expressed by LTi cells), IL-7R (expressed by LTi cells and their precursors), and CD45 (expressed by all hematopoietic cells) as described (26). Stainings were analyzed on a Leica TCS-SP2-AOBS Confocal Laser Scanning Microscope (Leica Microsystems) and images were obtained with Leica confocal software. Image processing involved contrast enhancement and region of interest selection, which was conducted with Jasc Paintshop Pro 7.0 (Jasc Software). Lenses used were dry lenses: 20× (HC PL APO CS 0.7), 40× (HCX PLAN APO 0.85).

Antibodies

For immunofluorescence, the following Abs were used: GK1.5 (anti-CD4), MECA-367 (MAdCAM-1), MP33 (anti-CD45), and anti-ICAM-1. All the Abs were affinity purified from hybridoma cell culture supernatants with protein G-Sepharose (Pharmacia) and biotinylated or labeled with Alexa-Fluor 488, Alexa-Fluor 546, or Alexa-Fluor 633 (Invitrogen Life Technologies). 429 (anti-VCAM-1; BD Biosciences), IK22/5 (anti-TRANCE; eBioscience), A7R34 (anti-IL-7R; eBioscience), 4H8WH2 (anti-LTβR; produced in Carl Ware's laboratory), HM0104 (anti-TNF-R1; Alexis Biochemicals), Avas12a1 (anti-VEGFr2; eBioscience), MECA-32 (pan-endothelial-cell marker; BD Biosciences), anti-Lyve-1 pAb (Millipore), 11D4.1 (anti- VE-cadherin; BD Biosciences), and anti-VEGFr1 pAb (anti-Flt1; Neomarkers) were used as biotinylated or as unconjugated primary Abs. 429, IK22/5, A7R34, Avas12a1, MECA-32, anti-Lyve-1 pAb, 11D4.1, and anti-VEGFr1 pAb were visualized with Alexa-Fluor 488, Alexa-Fluor 546, or Alexa-Fluor 633 conjugated streptavidin, anti-rat IgG or anti-rabbit IgG as appropriate. 4H8-WH2 and HM0104 were visualized with biotinylated anti-rat IgG, followed by signal amplification using a TSA Kit with HRP-streptavidin and Alexa Fluor 546 tyramide (Invitrogen Life Technologies). To assure specificity of the used Abs, conjugate-alone controls as well as control serum (rat or rabbit) as replacement of the primary incubation were used.

Mouse embryonic fibroblasts (MEFs) cell culture

MEFs from WT and $LT\beta R^{-/-}$ mice were established as described previously (27). Cultures were maintained in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stimulation of cells was performed by incubation with a mixture of two different agonistic anti-LTβR mAb (4H8-WH2 and 3C8 in a 9 to 1 proportion, with a total concentration of 2 μg/ml) or with 4H8-WH2 alone at 2 μg/ml or with isotype controls (Rat IgG2a and Rat IgG1, in the same proportion and concentration, BD Pharmingen). MEFs were stimulated for 2–30 h. ICAM-1, VCAM-1, and TRANCE induction on MEFs following LTβR stimulation was evaluated by flow cytometry at 24 h, using biotin-conjugated anti-ICAM-1 (eBioscience), FITC conjugated anti-VCAM-1 (eBioscience), and PE-conjugated anti-TRANCE mAb (eBioscience). Flow cytometric analysis was performed on a Cyan Advanced Digital Processing High-Performance Research flow cytometer (Beckman Coulter).

$LT\alpha^{-/-}$ rudimentary mesenteric LN (rMLN) cell culture

Embryos from $LT\alpha^{-/-}$ mice were used to dissect total E18.5 rMLN anlagen. Cell suspensions were prepared by digesting isolated rMLN anlagen with 0.5 mg/ml collagenase type IV (Sigma-Aldrich) in PBS, 2% NBCS for 20 min at 37°C while stirring continuously. Cell suspensions were cultured in DMEM (Invitrogen Life Technologies), supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Before stimulation, cells were allowed to adhere overnight at standard culture conditions to form a confluent layer of cells. For LTβR stimulation, cell suspensions were stimulated for 8 h with 4H8-WH2 (agonistic anti-LTβR Ab) (9, 28–30) at 2.68 μg/ml, while control samples were nonstimulated.

Quantitative real-time PCR

RNA was extracted from cultured MLN cell suspensions and MEFs using TRIzol (Invitrogen Life Technologies), and reverse transcribed with oligo(dT)12–18 (Invitrogen Life Technologies) and random hexamer primers (Invitrogen Life Technologies) using standard protocols. Quantitative real-time PCR was performed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The reaction mixture was composed of SYBR Green Mastermix (Applied Biosystems), 300 nM of each primer, and cDNA in a total volume of 20 μl, according to the manufacturer's instructions. Primers were designed across exon-intron boundaries using Primer Express software and guidelines (Applied Biosystems). The following sequences were used: 18S forward, TTGACGGAAGGGCACCACCAG, 18S reverse, GCACCACCACCCACGGAATCG; Hprt forward, CCTAAGAT GAGCGCAAGTTGAA, Hprt reverse, CCACAGGACTAGAACACCT GCTAA; GAPDH forward, GCATGGCCTTCCGTGTTC, GAPDH reverse, ATGTCATCATACTTGGCAGGTTTCT; Rps27a forward, AAGGTGGATGAAAATGGCAAA, Rps27a reverse, CCATGAAAA CTCCCAGCACCA; VCAM-1 forward, ACTACAAGTCTACATCTCT CCCAGGAAT, VCAM-1 reverse, CCTCGCTGGAACAGGTCATT; TRANCE forward, CCCATCGGGTTCCCATAAAG, TRANCE reverse, TAACCCTTAGTTTTCCGTTGCTTAA; IL-7 forward, ATCGTGCTG CTCGCAAGTT, IL-7 reverse, CACCAGTGTTTGTGTGCCTTGT; VEGF-C forward, GGTTACCTCAGCAAGACGTTGTTT, VEGF-C reverse, ATGCACCGGCAGGAAGTG.

From a set of eight housekeeping genes, the four most stable housekeeping genes were selected (18s, Hprt, GAPDH, and Rps27a) upon which a gene expression normalization factor for each tissue sample was calculated using geNorm 3.4 (http://medgen.ugent.be/~jvdesomp/genorm/) (31).

Statistics

Stimulated vs control cells from MLN anlagen were compared using an unpaired *t* test. Samples of MEFs were compared using a one-way ANOVA followed by a Tukey-Kramer multiple comparison test to allow comparison between different time points. p values of <0.05 were considered significant.

Results

Characterization of E14.5 LN anlagen

Because the very first events in LN development have not been fully characterized, we analyzed LN anlagen at different anatomical locations in E14.5 embryos for the presence of the various cellular subsets required for functional lymph nodes. In brachial LN anlage (Fig. 1*A*), axillary, iliac, and renal LN anlagen, LTi cells already form clusters, while a diffuse aggregation of LTi cells was observed in popliteal LN anlagen (supplementary Fig. 1).[4] In mesenteric LN anlagen, few LTi cells were situated around a large

[4] The online version of this article contains supplemental material.

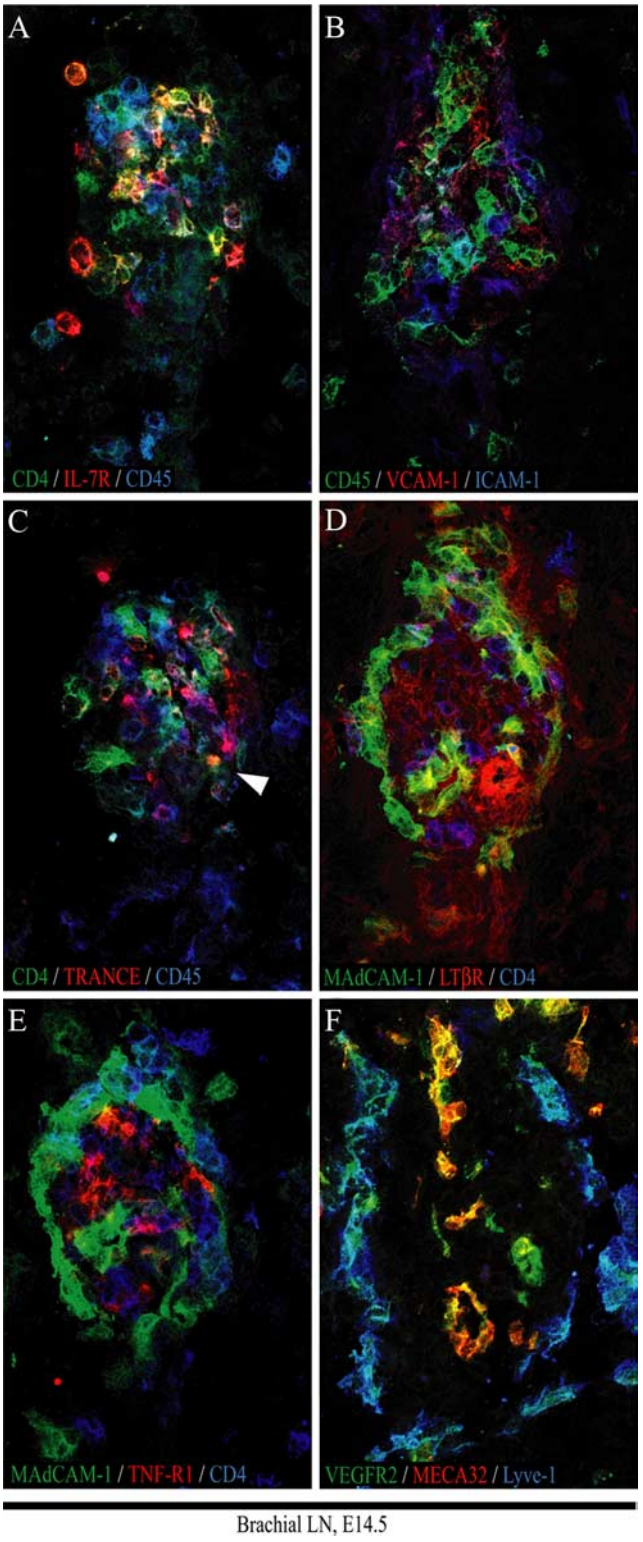

FIGURE 1. E14.5 WT BLN anlage characterization. *A*, $CD4^+IL\text{-}7R^+CD45^{int}$ and few immature $CD4^-IL\text{-}7R^+CD45^{int}$ LTi cells are present in the E14.5 anlage (CD4 in green, IL-7R in red, and CD45 in blue). *B*, VCAM-1 expression (red) on stromal cells is limited at this time point, but ICAM-1 expression (blue) can be found on blood vessels and $CD45^+$ hematopoietic cells (green). *C*, TRANCE expression (red) is limited to $CD45^+$ hematopoietic cells (blue) of which a number are $CD4^+$ LTi cells (green). Arrowhead points to TRANCE-expressing LTi cell (*D*) $LT\beta R^{int}$ and MAdCAM-1^+ stromal cells, $LT\beta R^+$ endothelial cells, and $CD4^+$ LTi cells (MAdCAM-1 in green, LTβR in red, CD4 in blue) occupy the E14.5 BLN anlage. *E*, MAdCAM-1^+ stromal cells (green) colocalize with $CD4^+$ LTi cells (blue) and TNF-$R1^+$ cells (red). *F,* LN anlagen contain

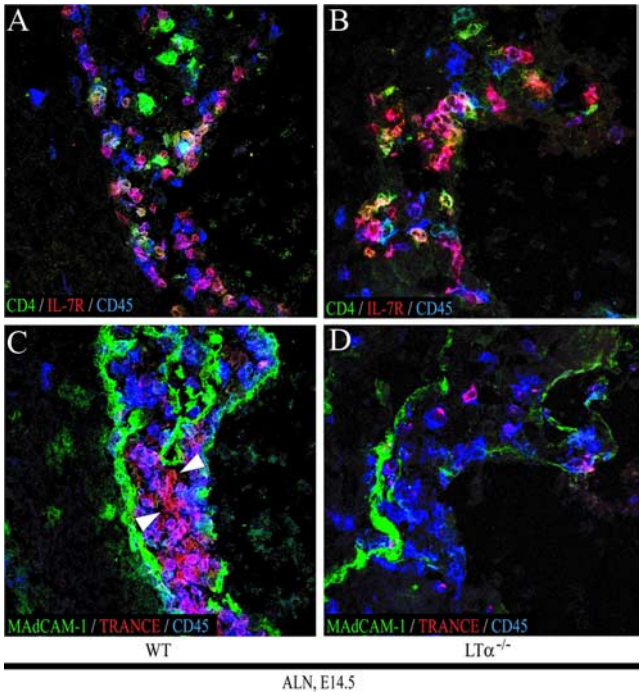

FIGURE 2. TRANCE expression in E14.5 WT Axillary LN and $LT\alpha^{-/-}$ rALN anlagen. *A* and *B*, ALN anlagen are found as clusters of hematopoietic cells in both WT and $LT\alpha^{-/-}$ at E14.5 (CD4 in green, IL-7R in red, and CD45 in blue). *C* and *D*, Subsequent sections were stained for MAdCAM-1 (green), TRANCE (red), and CD45 (blue). TRANCE expression is found on $CD45^+$ hematopoietic cells in both WT (*C*) and $LT\alpha^{-/-}$ (*D*) LN anlagen. Arrowheads indicate MAdCAM-1^+TRANCE$^+$ stromal cells. At E14.5, MAdCAM-1^+ cells surround TRANCE expressing cells in WT ALN anlagen and $LT\alpha^{-/-}$ rALN anlagen. *A–D* lenses used: ×40.

blood vessel. Inguinal LN anlagen could not be identified at E14.5. A considerable number of LTi cells expressed TRANCE at levels that varied from low to high (Fig. 1*C* arrowhead; and supplementary Fig. 1). Because we have previously reported that stromal cells within neonatal lymph nodes also express TRANCE, we were surprised that stromal cells within E14.5 anlagen of brachial lymph nodes did not show any expression of TRANCE, while the stromal cells showed limited expression of VCAM-1 and ICAM-1 (Fig. 1*B*). At E14.5, most $CD45^+$ clusters were encapsulated by Lyve-$1^+VEGFr2^+$ lymphatic endothelial cells and situated around a $VEGFr2^+MECA32^+$ blood vessel (Fig. 1*F*). In addition, in all LN anlagen, an additional larger blood vessel was found that consisted of $VEGFr1^+VEGFr2^+MECA32^{-/low}$ endothelial cells (data not shown). Combined expression of VEGFr1 and VEGFr2 was also observed on the aorta and not on the inferior vena cava at E14.5, suggesting that the $VEGFr1^+VEGFr2^+MECA32^{-/low}$ endothelial cells present within the lymph nodes represent an arterial blood vessel (supplementary Fig. 2). These $VEGFr2^+MECA32^{low}$ blood vessels also showed high expression of LTβR, while stromal cells of axillary (ALN) and brachial (BLN) anlagen expressed LTβR at an intermediate level (Fig. 1*D*). In addition, TNF-$R1^+$ cells could be found in E14.5 LN anlagen and these cells were in close association with $CD4^+$ cells (Fig. 1*E*).

$VEGFr2^+MECA32^{low}$ and $VEGFr2^+MECA32^+$ blood vessels and are surrounded by $VEGFR2^+$Lyve-1^+ lymphatic endothelial cells (VEGFr2 in green, MECA32 in red, Lyve-1 in blue). *A–F* lenses used: ×40.

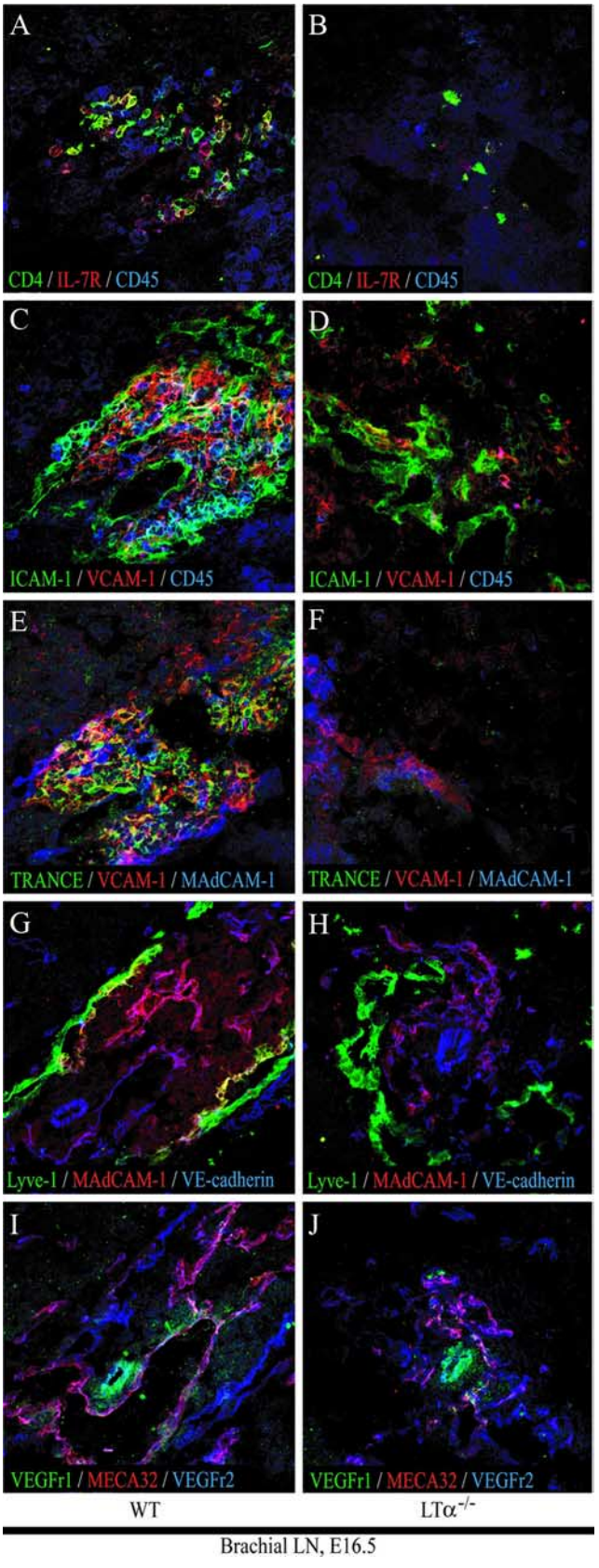

FIGURE 3. Altered morphology of E16.5 LT$\alpha^{-/-}$ BLN anlagen and lack of TRANCE expression on stromal cells. *A*, Clustered LTi cells are found in WT (*A*) BLN anlagen but not in LT$\alpha^{-/-}$ (*B*) BLN anlagen (CD4 in green, IL-7R in red, and CD45 in blue). At E16.5, large numbers of CD45$^+$ hematopoietic cells (blue) and ICAM-1$^+$VCAM-1$^+$ stromal cells (ICAM-1 in green, VCAM-1 in red) are found in the WT BLN anlage (*C*) but not in the LT$\alpha^{-/-}$ BLN anlage (*D*). ICAM-1$^+$VCAM-1^{low} vessels are found in both WT and LT$\alpha^{-/-}$ animals. TRANCE$^+$VCAM-1$^+$ and MAdCAM-1$^+$ stromal cells are found in WT BLN anlage (*E*) but not in LT$\alpha^{-/-}$ BLN anlagen (TRANCE in green, VCAM-1 in red, MAdCAM-1 in blue) (*G*). BLN anlagen contain Lyve-1$^+$VE-cadherin$^+$ LECs, MAdCAM-1^{int} stromal cells, VE-cadherin$^+$, and VE-cadherin$^+$MAdCAM-1$^+$ blood vessels (*H*). Both WT BLN anlagen (*I*) and LT$\alpha^{-/-}$ BLN anlagen (*J*) contain MECA32$^+$VEGFr2$^+$ blood vessels and a VEGFr1$^+$VEGFr2$^+$ blood vessel. In addition, VEGFr2$^+$ lymphatics are present at this time point in development (VEGFr1 in green, MECA32 in red, VEGFr2 in blue). *A–J* lenses used: ×40.

TRANCE expression in the first phase of LN development is LT$\alpha_1\beta_2$-LTβR independent

Because it has been reported that the earliest events in LN development occur independently of LTβR signaling (24, 25), we investigated whether the early clusters of LTi cells could indeed be found in E14.5 LT$\alpha^{-/-}$ embryos. In both WT and LT$\alpha^{-/-}$ embryos, LN anlagen contained clusters of LTi cells, of similar size (Fig. 2, *A* and *B*). However, a clear dissimilarity was seen when the expression of TRANCE was analyzed, because in WT mice, the first TRANCE-expressing stromal cells could already be observed within restricted areas of some LNs, in addition to the TRANCE expressing CD45$^+$ cells, while in LT$\alpha^{-/-}$ LNs, TRANCE expression was exclusively detected on CD45$^+$ cells, and not on stromal cells (Fig. 2, *C* and *D*). Of interest is that within WT LN anlagen TRANCE-expressing stromal cells are only present within areas where CD45$^+$ cells are clustered closely together, while absent at the location where the CD45$^+$ cells are more dispersed (Fig. 2*C*). Because stromal cell expression of TRANCE was completely absent in E14.5 LN anlagen of LT$\alpha^{-/-}$ embryos, these data suggest that TRANCE expression on stromal organizer cells depends on LTβR triggering, and that this occurs earliest at the site where LTi cells are closely together.

LT$\alpha^{-/-}$ LN anlagen at E16.5 lack stromal cell organizers and hematopoietic cells

To further dissect the role of LTβR signaling for stromal cell differentiation, we analyzed E16.5 LN anlagen because at this time point stromal cells are clearly expressing all differentiation molecules such as VCAM-1 and TRANCE (8). When WT and LT$\alpha^{-/-}$ anlagen were compared clear differences between WT and LT$\alpha^{-/-}$ embryos could now be observed in developing lymph nodes. At E16.5, large clusters of LTi cells were found in WT LN anlagen (Fig. 3*A*), whereas in most LT$\alpha^{-/-}$ LN anlagen studied only diffusely distributed LTi cells were found, with occasional small clusters of LTi cells (Fig. 3*B*). These data thus indicate that the clusters of LTi cells observed in E14.5 LT$\alpha^{-/-}$ embryos had greatly disappeared in the following 2 days of development. The remaining LTi cells in LT$\alpha^{-/-}$ LN anlagen were always located in proximity of VEGFr1$^+$VEGFr2$^+$Meca32^{low}-expressing vessels, indicative of proper positioning relative to the major blood vessel that is central to developing lymph nodes (Fig. 3, *B* and *J*). However, an intimate clustering as seen in WT lymph nodes was never detected in LT$\alpha^{-/-}$ LN anlagen, where stromal cells failed to express VCAM-1, ICAM-1, and MAdCAM-1 (Fig. 3, *C–H*). As expected from our observations at E14.5, TRANCE expression on stromal cells was completely absent in LT$\alpha^{-/-}$ PLN and MLN anlagen, further supporting the idea that TRANCE expression is controlled by LTβR signaling (Fig. 3, *E* and *F* and data not shown). The capsule of Lyve-1$^+$ expressing lymphatic endothelial cells (LECs) around the LN anlage, which forms a continuous layer in WT mice, appeared to form distinct lymph vessels around the LN anlagen in LT$\alpha^{-/-}$ mice (Fig. 3, *G* and *H*).

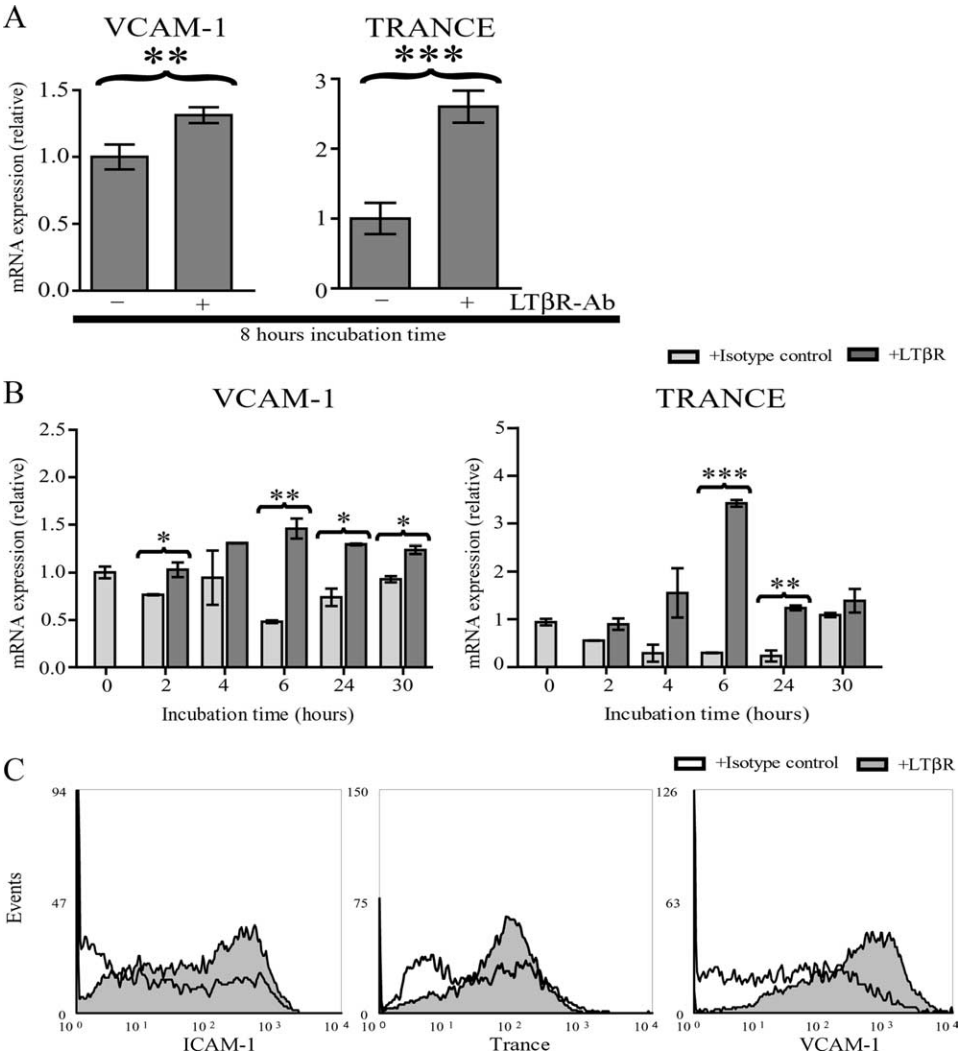

FIGURE 4. LTβR triggering results in TRANCE up-regulation (*A*). Stimulation of LTβR with agonistic anti LTβR mAb for 8 h in LTα$^{-/-}$ E18.5 rudimentary MLN cell cultures results in a significant increase in TRANCE mRNA expression compared with untreated cell cultures. Proper stimulation with the agonistic anti-LTβR mAb was validated by increase of VCAM-1 expression. Experiments were performed three times (*B*). Treatment of cultured WT MEFs with agonistic anti-LTβR mAb, but not with an isotype-matched control mAb, results in the up-regulation of TRANCE and VCAM-1 mRNA expression. MEFs were collected at 2, 4, 6, 24, and 30 h after stimulation for analysis of mRNA expression and (*C*) at 24 h after stimulation for analysis of ICAM-1, TRANCE, and VCAM-1 protein expression by FACS. Expression of transcripts in *B* was normalized to endogenous references genes as indicated. Relative expression levels at t = 0 were set at 1,0. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Although clear phenotypic differences could be found for LN development in WT vs LTα$^{-/-}$ embryos with respect to clusters of LTi cells and lack of LN stromal cells and LECs, the development of larger blood vessels within the designated area of LN formation remained unchanged in LTα$^{-/-}$ LN anlagen. In fact, the characteristic combination of VEGFr2^{+}MECA32^{+} vessels, which are always in close proximity to a vessel expressing VEGFr1 and VEGFr2, while lacking MECA32, allowed us to identify the LN regions within the LTα$^{-/-}$ embryos (Fig. 3, *I* and *J*).

LTβR triggering induces IL-7 and TRANCE in stromal cells

To prove that indeed signaling via the LTβR pathway directly controls TRANCE expression in stromal cells of the LN anlagen, in vitro experiments were performed. Cell suspensions from E18.5 LTα$^{-/-}$ rudimentary MLN anlagen were prepared, because these structures contain mesenchymal cells that have never encountered LTα$_1$β$_2$ expressing cells, which mediates their differentiation toward VCAM-1^{+}ICAM-1^{+} stromal organizer cells. Incubation for 8 h with an agonistic anti-LTβR Ab resulted in the expected increase of VCAM-1 measured by real time PCR, which served as a positive control for proper activation of the stromal cells (Fig. 4*A*) (9). Further analysis showed that indeed LTβR triggering resulted in the induction of TRANCE expression (Fig. 4*A*). To further confirm that LTβR can mediate the induction of TRANCE, MEFs were obtained from WT mice, representing early mesenchymal subpopulations. When incubated with the agonistic anti-LTβR Ab WT MEFs showed significant increase of TRANCE expression at 6 and 24 h of stimulation (Fig. 4*B*). A similar pattern of induction was seen for VCAM-1, as has been reported before (Fig. 4*B*) (9). Analysis of protein expression by FACS showed that indeed TRANCE, as well as VCAM-1, and ICAM-1 expression were induced on MEFs after 24 h of stimulation with agonistic anti-LTβR Ab (Fig. 4*C*).

The differentiation of mesenchymal cells toward stromal organizer cells creates a positive feedback loop through which incoming precursor cells are able to differentiate into true LTi cells, because TRANCE has been described to induce LTα$_1$β$_2$ expression on LTi cells (3). Another molecule that can mediate differentiation of precursors to fully mature LTi cells is the cytokine IL-7, which can also induce LTα$_1$β$_2$ expression on LTi cells. Therefore, LTβR-stimulated MEFs were analyzed for the expression of IL-7, which was indeed induced upon LTβR triggering in WT, but not in LTβR$^{-/-}$ MEFs (Fig. 5*A*). This suggests that LTβR triggering within lymph node anlagen results in differentiation of mesenchymal cells to functional stromal organizers cells, which are now able to induce the expression of LTα$_1$β$_2$ on newly arriving precursors to LTi cells.

To see whether continued LTβR triggering resulted in a further increase of TRANCE expression of stromal organizer cells, later stages of LN development were analyzed. Indeed, at E18.5 and at 1 day after birth lymph node stromal cells, characterized by expression of VCAM-1 and ICAM-1, showed high levels of

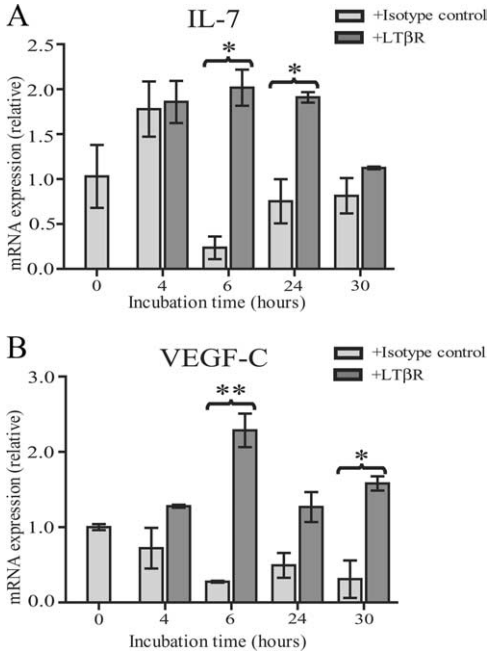

FIGURE 5. LTβR triggering leads to induction of IL-7 and VEGF-C. Treatment of cultured WT MEFs with agonistic LTβR mAb, but not with an isotype matched control mAb, results in the up-regulation of IL-7 (*A*) and VEGF-C (*B*) mRNA expression. MEFs were collected at 4, 6, 24, and 30 h after stimulation with agonistic LTβR mAb 4H8WH2. Expression of transcripts was normalized to endogenous references genes as indicated. Relative expression levels at t = 0 were set at 1,0. Experiments were performed three times. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

TRANCE expression, confirming our earlier observations (supplementary Fig. 3) (8).

LTβR triggering up-regulates lymphangiogenic factors in LN

Because we also observed alterations in lymphatic vessel formation in $LT\alpha^{-/-}$ vs WT and LTβR signaling has been implicated to be involved in lymphangiogenesis in inflamed areas (32), we further addressed whether lymphangiogenic factors were also induced by LTβR signaling. A strong up-regulation of VEGF-C by LTβR triggering was seen in MEF cultures (Fig. 5*B*). Up-regulation was first seen after 6 h of LTβR triggering and was still visible after 24 h of stimulation. $LT\beta R^{-/-}$ MEFs did not show increased expression of VEGF-C, which proved the specificity of the observed VEGF-C induction by LTβR triggering. We could not find a significant increase of VEGF-D expression in cultured MEFs upon LTβR triggering (data not shown).

Discussion

In this study, we show that during murine lymph node development the earliest clustering of LTi cells occurs independently of LTα and that the first TRANCE expressing stromal cells appear in LN anlagen from WT, and not $LT\alpha^{-/-}$, mice in areas where $CD45^+$ cells are closely clustered together. The interaction among the hematopoietic cells may lead to paracrine triggering, resulting in the first $LT\alpha_1\beta_2$ expressing cells. These cells are now able to trigger the surrounding stromal cells, which subsequently up-regulate TRANCE, IL-7, and VEGF-C, in addition to adhesion molecules and chemokines. The induced molecules will bring about further attraction and retention of more $LT\alpha_1\beta_2$ negative pre-LTi cells, and induction of $LT\alpha_1\beta_2$ expression on these pre-LTi cells, leading to further triggering of mesenchymal cells.

The earliest expression of TRANCE can be detected on LTi cells that arrive at the location of future LN development. This expression is independent of $LT\alpha_1\beta_2$-mediated LTβR signaling because in both WT and $LT\alpha^{-/-}$ mice LTi cells express TRANCE. It is very well likely that the paracrine triggering of the first pre-LTi cells that leads to the expression of $LT\alpha_1\beta_2$ is mediated through TRANCE and its receptor, as it has been shown that $LT\alpha_1\beta_2$ up-regulation can be accomplished by TRANCE-R signaling. Both TRANCE-R and TRANCE are expressed by the hematopoietic cells within the LN anlage (this study; Ref. 3, 18) and can thus account for the induction of the first $LT\alpha_1\beta_2$ expressing cells.

How these first hematopoietic cells are attracted to the designated locations is still an open question. Local expression of chemokines at designated sites of LN development might attract these "first wave" LTi cells, similar to the accumulation of LTi cells after ectopic expression of CXCL13 (15). It is however unclear at this point which factors may induce the expression of these chemokines.

After LTβR triggering, stromal cells begin to express adhesion molecules and chemokines (9). These molecules are required to retain the "first wave" LTi cells, but are also involved in the attraction of additional hematopoietic cells, containing precursor ($LT\alpha_1\beta_2$ negative) LTi cells that arrive in a "second wave". These cells will also be retained within the LN anlage, due to the adhesion molecules that are now being expressed by the stromal organizer cells. Retention of the earliest hematopoietic cells is not successful in the $LT\alpha^{-/-}$ mice, in which LN anlagen are devoid of hematopoietic clusters in E16.5 embryos. The mature stromal organizer cells in E16.5 WT embryos will allow local differentiation into mature $LT\alpha_1\beta_2$ expressing LTi cells. Induction of $LT\alpha_1\beta_2$ on these cells can be mediated by either TRANCE or IL-7, because both factors are expressed by stromal cells upon LTβR triggering. This results in a rapid increase of mature $LT\alpha_1\beta_2$-expressing LTi cells that will further contribute to the LTβR signaling. In addition, it was recently shown that LTβR triggering is mandatory for stromal cell proliferation and survival during the development of inguinal lymph nodes (25).

Of interest is our observation that stromal cells in developing lymph nodes (E17.5) express TRANCE, while this expression is not detectable in developing Peyer's patches at the same time (supplementary Fig. 4). At this point in development, stromal organizers within Peyer's patches express VCAM-1 and ICAM-1 in an LTβR-dependent manner (2). However, after the animals are born stromal cells within subepithelial dome of the Peyer's patches start to express TRANCE (supplementary Fig. 4). This agrees with the reported expression of TRANCE on stromal cells within the subepithial dome area of the Peyer's patches, intestinal isolated lymphoid follicles and cryptopatches from adult mice (33). In these studies it was shown that TRANCE expression in the intestine was independent of LTβR triggering, suggesting that TRANCE expression is controlled differentially during LN formation vs intestinal lymphoid structures in adult mice. The restricted expression of TRANCE in developing lymph nodes, and not Peyer's patches, matches with the fact that TRANCE expression is mandatory for LN formation, while it is redundant for Peyer's patch development (18).

A crucial role of LTβR signaling in the maintenance of functional high endothelial venules has been shown, highlighting the importance of this signaling route for blood vessel differentiation within lymph nodes (34). We show in this study that LTβR triggering results in increased expression of VEGF-C, further adding to the mechanism of how LTβR triggering results in LN formation. In this way, LTi cells can also contribute to lymphangiogenesis in

developing lymph nodes. Remodeling of lymph vessels also occurs in adult LNs as a result of immunization. This process might also be regulated by $LT\alpha_1\beta_2$ because lymphangiogenesis in inflamed LNs was shown to involve B cells that can also express $LT\alpha_1\beta_2$, and lymphangiogenesis in inflamed thyroid glands was shown to depend on LTβR signaling (32, 35, 36).

In sum, our experiments show that the first phase of LN development occurs similarly in WT and $LT\alpha^{-/-}$ mice, and that subsequent LTβR triggering leads to the induction of TRANCE and IL-7, which can further enhance $LT\alpha_1\beta_2$ expression. In addition, LTβR triggering can also lead to the production of the lymphangiogenic factor VEGF-C. As a consequence, these LN stromal organizers may control attraction, retention, as well as lymphangiogenesis to the developing lymph nodes.

Disclosures

The authors have no financial conflict of interest.

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