Endothelial cell–specific lymphotoxin-β receptor signaling is critical for lymph node and high endothelial venule formation

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The development of lymph nodes (LNs) and formation of LN stromal cell microenvironments is dependent on lymphotoxin-β receptor (LTβR) signaling. In particular, the LTβR-dependent crosstalk between mesenchymal lymphoid tissue organizer and hematopoietic lymphoid tissue inducer cells has been regarded as central for these processes. Here, we assessed whether endothelial cell (EC)–restricted LTβR signaling impacts on LN development and the vascular LN microenvironment. Using EC-specific ablation of LTβR in mice, we found that conditionally LTβR-deficient animals failed to develop a significant proportion of their peripheral LNs. However, remnant LNs showed impaired formation of high endothelial venules (HEVs). Venules had lost their cuboidal shape, showed reduced segment length and branching points, and reduced adhesion molecule and constitutive chemokine expression. Due to the altered EC–lymphocyte interaction, homing of lymphocytes to peripheral LNs was significantly impaired. Thus, this study identifies ECs as an important LTβR-dependent lymphoid tissue organizer cell population and indicates that continuous triggering of the LTβR on LN ECs is critical for lymphocyte homeostasis.

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organization with luminal localization of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), which functions as anchors for cells circulating in the blood (Tohya et al., 2010). LTβR signals are important for maintenance of the HEV network under homeostatic conditions, as demonstrated by systemic treatment of adult mice with an LTβR decoy receptor (Browning et al., 2005). Furthermore, myeloid lymphotxin-expressing cells, such as dendritic cells, can interact with HEV ECs to stimulate their maturation (Moussion and Girard, 2011). However, other LN stromal cells, including fibroblastic reticular cells (FRCs), can be efficiently triggered via the LTβR to secrete potent vascular growth factors, establishing a multicellular regulation circuit that maintains the homeostasis of the HEV network (Chyou et al., 2008). Thus, it has remained elusive whether the generation and maintenance of HEV morphology and function is determined by direct LTβR signals in ECs or whether LTβR-dependent communication between different LN stromal cell populations generates the appropriate microenvironment.

To assess the impact of specific and constitutive ablation of LTβR signaling in ECs, we crossed vascular endothelial cadherin (VE-cadherin)-Cre mice (Alva et al., 2006) with Ltb rf/fl mice (Wimmer et al., 2012). We found that the specific deletion of the LTβR on ECs blocked the development of a significant proportion of peripheral LNs. Furthermore, EC-specific LTβR signaling was critical for formation of the HEV network and control of lymphocyte trafficking.

RESULTS AND DISCUSSION

Targeting of LTβR-expressing ECs with the VE-cadherin-Cre transgene

LTβR is broadly expressed in various tissues of the developing mouse embryo (Browning and French, 2002), and LTβR deficiency severely impairs the development of peripheral lymphoid tissues (Fütterer et al., 1998). To assess the LTβR expression pattern in major LN stromal cell populations, we separated CD45+ stromal cells by FACS sorting using the well-established markers podoplanin and CD31 (Malhotra et al., 2012) into Pdpn−CD31+ blood ECs (BECs), Pdpn−CD31+ lymphatic ECs (LECs), and Pdpn−CD31+ FRCs (Fig. 1 a). Mesenchymal versus endothelial lineage identity of the sorted cell populations was confirmed by exclusive Wnt1 mRNA expression in the FRC fraction (Fig. 1 b). Ltb mRNA expression was comparable in the three major LN stromal cell populations (Fig. 1 c), whereas LTβR expression on the surface of both BECs and LECs was significantly higher compared with FRCs (Fig. 1, d and e) suggesting that ligation of the LTβR on ECs may precipitate important functional changes in ECs.

To assess the developmental and functional consequences of LTβR ablation in ECs, we used transgenic VE-cadherin-Cre mice. In these mice, VE-Cadherin-Cre activity starts in the embryonic endothelium at E8, i.e., before the initial steps of LN development (Alva et al., 2006). At E16.5, transgene-expressing ECs co-localized with CD45+ LTi cells in the anlage of the inguinal LN (Fig. 2 a). Importantly, transgene activity in developing inguinal LNs (E18.5) of VE-Cadherin-CreR26-RFP mice was not associated with CD45+ cells (Fig. 2 b). Likewise, in neonatal LNs, transgene expression was confined to CD31+ ECs (Fig. 2 c). Importantly, ECs in the neonatal LN coexpressed LTβR and the VE-cadherin-Cre transgene, with the highest abundance in the region of the subcapsular sinus (Fig. 2 c, i–iv). Confocal microscopic (Fig. 2 d) and flow cytometric (Fig. 2 e) analysis of adult LNs from VE-cadherin-CreR26-RFP mice confirmed that ~70% of LN LECs and BECs express the RFP reporter, indicating that the VE-cadherin-Cre transgene targets a major proportion of LTβR-expressing LN ECs.

Impact of EC-specific LTβR ablation on LN formation and structure

To ablate the LTβR on ECs, we crossed the VE-cadherin-Cre transgene onto the Ltb rf/fl background (Wimmer et al., 2012). As shown in Fig. 3 a, VE-cadherin-CreLtb rf/fl mice failed to...
As a consequence, the size of B cell follicles (Fig. 3 h), as well as overall cellularity (Fig. 3 i), were reduced. Furthermore, the OPT analysis revealed a profound structural deficiency in the vasculature, i.e., a severely impaired MECA-79+ HEV network (Fig. 3 f). Quantification of HEV parameters revealed that the overall length of the MECA-79+ vascular structures (Fig. 3 j) and the mean number of branching points (Fig. 3 k) were reduced by >60% and >70%, respectively.

The exclusive impact of the conditional LTBR deficiency on ECs is shown by the preserved LTBR expression on FRCs, whereas both BECs and LECs in LNs of VE-cadherin-CreLtbfrfl/fl mice exhibited a significantly reduced LTBR expression (Fig. 3, l and m). In contrast to the profound effects on peripheral LNs, formation of the mesenteric LN was not affected by the EC-specific LTBR deficiency (Fig. 3, a and d). Those peripheral LNs that had succeeded to develop in VE-cadherin-CreLtbfrfl/fl mice appeared smaller, as shown in Fig. 3 e for an inguinal LN. Optical projection tomographical (OPT) analysis of inguinal LNs confirmed the significant size differences between conditionally LTBR-deficient and control LNs (Fig. 3, f and g).

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Figure 3. Ablation of LTβR signaling in ECs impacts peripheral LN development. (a) The presence of the indicated LNs was recorded in 4-wk-old VE-cadherin-CreLtβRfl/o mice and plotted as percent present/absent (n = 10 mice). Mice were injected with 1% Chicago sky blue 1 wk before the analysis. Microphotographs show presence or absence of (b) axillary (white arrow head) and brachial (black arrow head), (c) inguinal, or (d) mesenteric LNs in the indicated mouse strains. Representative microphotographs from one out of four mice per group. (e) Macroscopic appearance of developing inguinal LNs in VE-cadherin-CreLtβRfl/o mice (top image) and LTβR-proficient controls (bottom image). (f) Inguinal LNs from the indicated strains were analyzed by OPT for the presence of the HEV network (MECA-79+) and B cell follicles (B220+). Bar, 500 µm. Quantitative analysis based on OPT data for LN volume (g) and B cell follicle volume (h). (i) Flow cytometry–based quantification of LN cellularity. Values indicate relative cell numbers in VE-cadherin-CreLtβRfl/o mice compared with LtβR+/+ mice (mean ± SEM; n = 6 mice per group in 3 independent experiments). Quantitative analysis based on OPT data for (j) HEV network length and (k) HEV branching points (mean ± SEM; n = 6 mice per group pooled from three independent experiments). (l) Representative histograms showing flow cytometric analysis of LTβR expression on CD31+Pdpn+ LECs, CD31+Pdpn− BECs, and CD31−Pdpn+ FRCs of VE-cadherin-CreLtβRfl/o and LtβR+/+ LNs. (m) Quantification of MFI for LTβR expression in respective populations (mean ± SEM from six mice per group, pooled from two independent
of the EC-specific LTβR ablation on the LN vasculature, the formation of intranodal lymphatic structures was less severely affected in the absence of EC-specific LTβR signaling (Fig. 3, n and o). Likewise, lymphatic skin drainage toward the popliteal LN and intermodal lymph flow from the popliteal to the inguinal LN was not affected in VE-cadherin-CrexLtβRfl/fl mice (Fig. 3 p). Collectively, these data suggest that the VE-cadherin-Cre transgene targets an early EC population that is LTβR dependent and is critical for LN development. Furthermore, in those adult LNs that succeeded in their development, likely due to the incomplete targeting of ECs in VE-cadherin-Cre mice (i.e., ∼70% of all LN BECs and LECs), a pronounced impact of direct LTβR signaling in ECs was evident.

**Constitutive LTβR signaling in ECs is required for HEV formation and function.** To further determine the consequences of EC-specific LTβR deficiency, we performed high-resolution confocal microscopy and assessed the phenotypical changes in HEV ECs. Despite the absence of LTβR signaling, ECs had maintained some MECA-79 expression (Fig. 4 a). Furthermore, the ERTR-7+ FRC network surrounding the vascular structures was maintained (Fig. 4 a, arrowheads). However, the endothelium had lost its cuboidal shape and polarization, with decreased ICAM-1 staining on the luminal side (Fig. 4 a, arrow) resulting in a significantly reduced overall ICAM-1 expression (Fig. 4 b). Likewise, MECA-79+ ECs in the conditionally LTβR-deficient mice had lost expression of CCL21 (Fig. 4 c, arrows, top left), whereas stromal cells surrounding the blood vessel still produced CCL21 as visualized in three-dimensional reconstructions of the paracortical stromal network (Fig. 4 c, right). Consistent with this finding, we found that the selective ablation of LTβR on ECs resulted in a reduction of Cdx21 and Cdx19 mRNA expression in total LN tissue (Fig. 4 d). Furthermore, expression of the HEV addressin Glycam1 was almost completely lost in LNs of VE-cadherin-CrexLtβRfl/fl mice, whereas the expression of the follicular dendritic cell marker Mge8 was not affected (Fig. 4 e). Collectively, the MECA-79+ blood vessels in VE-cadherin-CrexLtβRfl/fl mice had lost typical properties of HEVs.

To assess whether the structural and phenotypical alterations in MECA-79+ blood vessels in VE-cadherin-CrexLtβRfl/fl mice would impact on lymphocyte migration, we adoptively transferred dye-labeled lymphocytes and assessed their LN-homing behavior in VE-cadherin-CrexLtβRfl/fl and control mice. As shown in Fig. 5 a, lymphocytes were still able to home to the LN parenchyma in VE-cadherin-CrexLtβRfl/fl mice. However, flow cytometry-based quantification revealed that homing of both T and B cells was strongly affected by the EC-specific LTβR deficiency (Fig. 5 b). High-resolution microscopic analysis showed that adoptively transferred lymphocytes could be found within the HEVs of LTβR-competent mice (Fig. 5 c, bottom, arrows), whereas those lymphocytes that had successfully entered LNs of VE-cadherin-CrexLtβRfl/fl mice were found exclusively in the LN parenchyma (Fig. 5 c, top, arrowheads). A recent study has shown that retention of lymphocytes in HEV pockets is an important regulatory step for lymphocyte trafficking (Mionnet et al., 2011). Indeed, in all LNs of LTβR-proficient mice, we found aggregates of CD4+ T cells surrounded by the MECA-79+ endothelium of HEVs (Fig. 5 d, bottom, arrows). However, this retention function was missing in the flat MECA-79+ endothelium of LNs from VE-cadherin-CrexLtβRfl/fl mice (Fig. 5 d, top). The CCR7 ligands CCL19 and CCL21 are important for the regulation of general T cell motility (Förster et al., 2008). Because we observed a general down-regulation of CCL21 and CCL21 in LNs of VE-cadherin-CrexLtβRfl/fl mice, we used intravital two-photon microscopy to determine whether general T cell motility would be affected by the EC-specific LTβR deficiency. As shown in Fig. 5 e, the specific LTβR defect in ECs and the resulting structural and functional alteration in the HEV network did not significantly impact on general T cell motility, neither in the T cell zone nor in the direct vicinity of MECA-79+ vessels. Collectively, constitutive LTβR signaling in LN vascular ECs appears to be necessary for the differentiation of vascular ECs into the typical HEV EC and, hence, the appropriate formation of the HEV network.

The earliest steps in LN development involve the PROX1-dependent metamorphosis of venous ECs to lymphatic ECs at the anterior cardinal vein (Srinivasan et al., 2007). These early LECs form the lymph sac which serves as the primordial tissue for both the LN anlagen and the sprouting lymphatic system (Blum and Pabst, 2006). After these initial steps, mesenchymal LTo and hematopoietic LTi cells are recruited to the LN anlage and the subsequent developmental steps are dependent on the constitutive chemokine receptor ligands CXCL13, CCL19, and CCL21 (Luther et al., 2003). Furthermore, triggering of the CD127/IL-7Rα on LTo cells in concert with constitutive chemokine signals is critical for the formation of LNs and Peyer’s patches. It has been suggested that LTβR signaling in mesenchymal LTo cells is the critical event that steers the cascading induction of constitutive chemokines and IL-7 (van de Pavert and Mebius, 2010). This particular view has been supported by the finding that the absence of lymphatic endothelium in PROX1-deficient mice did not block LN anlage formation (Vondenhoff et al., 2009). The present study challenges the concept that there is only one dedicated LTβR-dependent stromal LTo cell...
LEC- versus BEC-specific ablation of the LTβR will reveal which of the two EC populations function as an endothelial LTo cell. ECs and hematopoietic cells share the hemangioblast as a common precursor cell (Domigan and Iruela-Arispe, 2012) and therefore, some molecular traits are shared between the lineages. For example, VE-cadherin is involved in endothelial homotypic cell adhesion, a function that is critical for vascular development and function (Carmeliet et al., 1999). Consequently, the VE-cadherin-Cre transgene exhibits uniform expression in the endothelium of developing and quiescent vessels (Alva et al., 2006). However, a small proportion of hematopoietic cells express the LTβR and exhibit some functional changes after LTβR triggering. Nevertheless, because macrophage-specific ablation of the LTβR population. We found that the ablation of LTβR expression in ECs in VE-cadherin-CreLtbr<rfl/fl> mice led to impaired LN development. Because the VE-cadherin-Cre transgene is active in ECs but not in mesenchymal stromal cells (Alva et al., 2006; this study), our study provides conclusive evidence that LTβR signaling in endothelial LTo cells is mandatory for LN formation. At this point, we can only speculate at which stage of LN development LTo cells interact with early endothelial LTβR+ cells. It is possible that lymphotixin-expressing LTi cells stimulate LTβR+ LECs in the early LN anlage to produce more CCL21, and thereby contribute to the CCR7 dependency of LN development (Luther et al., 2003). Moreover, the development of the intranodal blood vasculature may be dependent on LTβR signals provided to BECs. Insufficiently developed LN blood vessels may restrict LN formation because growing tissues are particularly vulnerable to impaired blood supply. Future studies of conditional and inducible LEC- versus BEC-specific ablation of the LTβR will reveal which of the two EC populations function as an endothelial LTo cell.

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B cell–derived lymphotoxin significantly contributes to the remodeling and adaptation of the HEV network during the acute phase of a noncytopathic viral infection (Kumar et al., 2010). It has also been suggested that restructuring of the vascular LN microenvironment can be mediated by dendritic cells (Chyou et al., 2011). Likewise, the LTβR-dependent, homeostatic control of the adult LN HEV network (Browning et al., 2005) may be a result of multicellular processes (Chyou et al., 2008). The results of this study indicate that direct triggering of the LTβR on vascular ECs is important for the development of the cuboidal HEV appearance and the acquisition of lymphocyte traffic–regulating properties such as polarized ICAM1 expression, production of CCL21, and formation of pouches for lymphocyte retention. These findings bear interesting implications for the formation of tertiary lymphoid tissues in the course of infections and autoimmune diseases:

Figure 5. LTβR signaling in ECs is required for lymphocyte homeostasis. C57BL/6 splenocytes were labeled with CFSE and adoptively transferred into VECadherin-CreLtbβfl/fl mice and Ltbβ+/- mice. (a) Popliteal LN sections of indicated recipients were stained with fluorescently labeled antibodies against CD31, CD4, and CFSE and analyzed by confocal microscopy. Bar, 100 µm. (right) Higher magnification images of the boxed areas in the images on the left. Bar, 50 µm. Data show one representative analysis out of four. (b) Flow cytometric analysis of transferred cells (CFSE+) lymphocytes in VECadherin-CreLtbβfl/fl and Ltbβ+/- popliteal LNs. Left panel shows absolute numbers and right panel shows percentage of CFSE+ cells in the respective population (mean ± SEM from 4 mice pooled from two independent experiments). (c) High-resolution analysis of CFSE+ lymphocytes in (arrow) and around (arrowhead) CD31+ vascular endothelia in LNs of VECadherin-CreLtbβfl/fl and Ltbβ+/- recipients. Bar, 10 µm. (d) Confocal microscopic analysis of intraendothelial pocket formation (arrow) in LNs of VECadherin-CreLtbβfl/fl and Ltbβ+/- recipients. Bar, 10 µm. (e) Lymphocyte motility in the T cell zone and around HEVs in popliteal LNs of VECadherin-CreLtbβfl/fl and Ltbβ+/- mice as determined by intravital two-photon microscopy. Values represent single tracks pooled from two independent experiments (n = 4 mice per group, mean indicated by horizontal bar).

Concluding remarks
The characterization of the developmental pathways of LN organogenesis is most helpful for understanding of the primary functions of these immune compartments in the adult, namely concentration of pathogens and immune cells to secure swift activation of innate and adaptive immunity (Junt et al., 2008). During immune activation, LNs undergo profound morphological changes to optimally accommodate or restore particular interaction compartments. For example, the LTβR-dependent interaction of adult LTi cells with T cell zone FRCs is critical for the restoration of immunocompetence after viral infection (Scandella et al., 2008). Furthermore, did not affect LN formation (Wimmer et al., 2012), we conclude that the defect in LN development in VECadherin-CreLtbβfl/fl mice is a direct result of LTβR deficiency in ECs.
chronically activated, lymphotxin-expressing lymphocytes may directly induce the differentiation of ECs into the HEV phenotype, hence recapitulating the generation of the vascular LN microenvironment for optimal lymphocyte trafficking (Hayasaka et al., 2010). Further molecular dissection of this pathway may help to identify targets that attenuate the formation of tertiary lymphoid tissues at sites of chronic inflammation.

**MATERIALS AND METHODS**

**Mice.** B6.Cg-Tg(Cdh5-cre)7Mia/J (VE-Cadherin-Cre; Alva et al., 2006) and B6.129X1-Gt(Rosa)26Sortm148(Tg26-RFP) mice were purchased from The Jackson Laboratory. Ltbrfl/fl mice were described previously (Wimmer et al., 2012). C57BL/6 mice were obtained from Charles River. All animals were kept under conventional conditions in individually ventilated cages. Experiments were performed in accordance with federal and cantonal guidelines (Tierschutzgesetz) under the permission numbers SG11/05 and SG11/04 granted by the Veterinary Office of the Canton of St. Gallen.

**CFSE-labeling and adoptive transfer of cells.** Single-cell suspensions from spleens of C57BL/6 mice were subjected to hypotonic red blood cell lysis and stained with CFSE (Molecular Probes). A maximum concentration of 2.5 x 10^6 cells/ml were incubated in 5 µM CFSE in PBS for 10 min at 37°C. Cells were washed twice with ice-cold balanced salt solution (BSS) and resuspended in BSS. VE-Cadherin-Cre Lsdb/β- or control mice were injected intravenously with 10^7 C57BL/6 splenocytes in 200 µl BSS. Homing of transferred cells was analyzed 2 h after transfer by flow cytometry and histology.

**Preparation of stromal cells.** LNs were dissected into small pieces and transferred into a 24-well dish filled with RPMI 1640 medium containing 10% FCS, 1 mg/ml Collagenase Type IV (2% FCS, 20 mM Hepes (all from Lonza), 1 mg/ml Collagenase Type IV transferred into a 24-well dish filled with RPMI 1640 medium containing 10% FCS, 1 mg/ml Collagenase Type IV)

**Flow cytometry and cell sorting.** Single-cell suspensions were incubated for 20 min at 4°C in PBS containing 1% FCS and 10 mM EDTA with the following fluorescently labeled antibodies: anti-CD45, anti-gp38/podoplanin, anti-CD45, anti-B220, conjugated anti-CD31, anti-Lyve1 (eBioscience), anti-CD4, anti-ICAM-1 (BioLegend), and anti-MECA-79. Unconjugated antibodies were detected with the following secondary antibodies: DyLight649-conjugated anti-rat-IgG, Alexa Fluor 488-conjugated anti-rabbit-IgG, DyLight549-conjugated anti-Syrian hamster-IgG, and DyLight549-conjugated Streptavidin (all purchased from Jackson Immunoresearch Laboratories). Fluorescence signal of LTBR staining was amplified by using a tyramide- amplification kit (Molecular Probes) according to the manufacturer’s protocol. Microscopic analysis was performed using a confocal microscope (LSM-710; Carl Zeiss) and images were processed with ZEN 2010 software (Carl Zeiss). For tracing of lymphatic flow, mice were injected with 50 µg of 40-kD FITC-dextran into the hind footpad. 10 min after injection, mice were sacrificed and draining LNs were carefully excised under the stereomicroscope, fixed, and cut with a vibratome (VT-1200; Leica). FITC fluorescence per LN section was quantified using the LSM-710 confocal microscope.

**OPT.** Mice received an intravenous injection of fluorescently labeled MEGA-79 (12–15 µg) to label the HEV network. After 15 min, mice were sacrificed, LNs were carefully excised, and surrounding tissue was removed under a stereomicroscope. Sample preparation and OPT were performed as previously described (Kumar et al., 2010).

**Intravital microscopy.** Purified C57BL/6 T cells were fluorescently labeled with 2.5 µM chloromethyl-benzoyl amino-tetramethylrhodamine or CFSE for 15 min at 37°C. After washing, labeled T cells were injected intravenously into sex-matched mice, which were anesthetized and surgically prepared to expose the right popliteal LN. Immediately after injection, two-photon microscopy was performed as previously described (Sorrenti et al., 2011).

**Statistical analysis.** Two-photon microscopy and OPT data were analyzed with Velocity (Perkin Elmer) and Imars (Biplane). All statistical analyses were performed with Prism 5.0 (GraphPad Software Inc.). Data were analyzed with the nonparametric Student’s t-test. A p-value of <0.05 was considered significant.

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Author contributions: B. Ludewig designed the study and wrote the paper; L Onder performed research and wrote the paper; E. Scandella, J.V. Stein, and T. Hehlgans performed research and wrote the paper; E. Scandella, J.V. Stein, and T. Hehlgans analyzed data; R. Danuser, S. Finer, and Dan Chai performed research.

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