Lymph sacs are not required for the initiation of lymph node formation

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Development. 2009 Jan;136(1):29-34.
Abstract

The lymphatic vasculature drains lymph fluid from the tissue spaces of most organs and returns it to the blood vasculature for recirculation. Before reaching the circulatory system, antigens and pathogens transported by the lymph are trapped by the lymph nodes. As proposed by Florence Sabin more than a century ago and as recently validated, the mammalian lymphatic vasculature has a venous origin and is derived from primitive lymph sacs scattered along the embryonic body axis. Also as proposed by Sabin, it has been generally accepted that lymph nodes originate from those embryonic primitive lymph sacs. However, we now demonstrate that the initiation of lymph node development does not require lymph sacs. We show that lymph node formation is normally initiated in E14.5 Prox1-null embryos devoid of lymph sacs and lymphatic vasculature and in E17.5 Prox1–conditional mutant embryos, which have defective lymph sacs. However, subsequent clustering of hematopoietic cells within these developing lymph nodes is less efficient.
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Introduction

More than a century ago, Florence Sabin proposed a model for the development of the mammalian lymphatic vasculature. According to this model, endothelial cells bud from the veins to form primitive lymph sacs. From these sacs, lymphatic endothelial cells (LECs) sprout and form the entire lymphatic vasculature network. Initial support for the venous origin suggested by Sabin’s pioneering work was provided by the analysis of the expression of VEGFR3/Vegf-C, Prox1, and other genes whose activity is crucial for the formation of the entire lymphatic vasculature in mammalian embryos. In the case of Prox1, this gene’s activity is necessary and sufficient for the specification of the LEC phenotype in venous endothelial cells in vivo and in vitro. Sabin’s original venous model was recently validated by using a genetic lineage-tracing approach.

In addition to proposing a venous origin for the mammalian lymphatic vasculature, Sabin proposed that lymph nodes (LNs) originate from the embryonic primitive lymph sacs. A century later, this dogma has remained unchallenged despite the recent availability of valuable molecular markers and mouse models, and it is generally accepted that LNs require lymph sacs for their formation.

In this paper, we address this important question by taking advantage of mouse models in which Prox1 functional activity is either nullizygous, hemizygous, or conditionally removed from venous LEC progenitors. In these mutant mice, lymph sacs are either absent or defective; therefore, these animal models are a valuable asset in which to evaluate whether lymph sacs are required for LN formation.

We now conclude that primitive lymph sacs are not necessary for the initial formation of the mammalian LN anlagen; however, further progression into tight clusters of hematopoietic cells that interact with stromal cells appears to be sensitive to the presence of LECs and/or relatively normal lymph sacs.

Materials and Methods

Mice

C57BL/6 mice were purchased from Harlan (Horst, The Netherlands); lymphotoxin-alpha–deficient (LTα−/−) mice were purchased from Charles River (Maastricht, The Netherlands). Generation of Prox1+/−, Prox1−/−, Tie-2-Cre, and Prox1floxflox mice was previously reported. All animal experiments were approved by the local animal experimentation committee.

Mice were mated overnight, and the day of vaginal-plug detection was noted as embryonic day (E) 0.5. Pregnant females were killed at different time points, and embryos were harvested and prepared for sectioning by embedding and freezing.
Immunofluorescence
Following cryosections (7-μm thick) of the embryos, sections were fixed in dehydrated acetone for 2 min and then air dried for 15 min. Endogenous avidin was blocked with an avidin-biotin block (Vector Laboratories, Burlingame, CA, USA). Sections were then preincubated in PBS supplemented with 5% (v/v) mouse serum for 10 min. Incubation with the primary antibody for 45 min was followed by incubation with Fluor-Alexa–labeled conjugate (Invitrogen Life Technologies, Breda, The Netherlands) for 30 min. All incubations were carried out at room temperature. Sections were counterstained with Hoechst 33342 (Invitrogen Life Technologies) for 10 min and analyzed on a Leica TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems Nederland b.v., Rijswijk, The Netherlands).

Antibodies
The antibodies GK1.5 (anti-CD4), MECA-367 (anti–mucosal addressin cell adhesion molecule-1 [MAdCAM-1]), MP33 (anti-CD45), 8.1.1 (anti-podoplanin), and ERTR7 were affinity purified from the supernatants of hybridoma cell cultures by using protein G-Sepharose (Pharmacia, Uppsala, Sweden). The antibodies were biotinylated or labeled with Alexa-Fluor 488, Alexa-Fluor 546, or Alexa-Fluor 633 (Invitrogen Life Technologies). The antibodies A7R34 (anti-Il7r; eBioscience, San Diego, CA, USA), 429 (anti–VCAM-1; eBioscience), Avas12a1 (anti–VEGFR2; eBioscience), MECA32 (pan-endothelial-cell marker; BD Biosciences, Erembodegem, Belgium), anti–Lyve-1 (Millipore, Billerica, MA, USA), 11D4.1 (anti–vascular endothelial (VE)-cadherin; BD Biosciences, Erembodegem, Belgium), anti–VEGFR1 (anti-Flt1; Neomarkers, Fremont, CA, USA), AFL4 (anti-VEGFR3; eBioscience), anti-Prox1 (ReliaTech GmbH, Braunschweig, Germany), anti–RORγ(t) (kindly provided by D. Littman) , and anti–β-galactosidase (MP Biomedicals, Aurora, Ohio, USA) were used biotinylated or unconjugated and visualized with Alexa-Fluor 488, Alexa-Fluor 546 or Alexa-Fluor 633–conjugated streptavidin, anti–rat IgG or anti–rabbit IgG, or anti-Armenian hamster-Cy3 as appropriate.

Results and Discussion
To determine the origin of mammalian LNs, we first assessed the contribution of LECs to the developing LNs on E14.5 and E16.5 wild-type mouse embryos. It is well established that early during LN organogenesis and upon their interaction with lymphotxin-expressing hematopoietic lymphoid tissue inducer (LTI) cells, lymphotxin-beta receptor (LTβR)–expressing mesenchymal cells differentiate into specialized stromal organizer cells. However, before this LTβR-dependent process initiates, some type of signal induces the accumulation of LTi cells and stromal cells . Until now, the inductive signals from the primitive
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lymph sacs or differentiating LECs were considered the primary candidates in the process that initiates the clustering of LTi cells and stromal cells.

To characterize the presence of LECs at the site of LTi cell clusters, we immunostained wild-type LN anlagen with antibodies against CD4, which is expressed by LTi cells \(^{16}\); Il7r, which is expressed by LTi cells and their precursors \(^{15, 17}\); and CD45, which is expressed by all hematopoietic cells. This analysis revealed that approximately 50% of the CD45\(^+\) cells in the anlagen corresponded to LTi cells, as indicated by their expression of CD4 and Il7r at E14.5 (Fig. 1A). Cluster size increased between E14.5 and E16.5 (Fig. 1B). At E14.5, all of the LN anlagen were present in the embryo; however, the inguinal and popliteal LN anlagen consisted of very small clusters of LTi cells (data not shown). Therefore, the anlagen containing larger clusters of LTi cells (i.e., axillary, brachial, renal, cervical, mesenteric, thymic, and aortic) were used for detailed analysis.

To identify LECs within the LN anlagen, we immunostained adjacent sections with the stromal and endothelial markers MAdCAM-1, VE-cadherin, VEGFR1, VEGFR2, MECA32 and with the LEC markers Lyve-1, podoplanin, Prox1, and VEGFR3 \(^{2, 5, 16 - 21}\). At E14.5, the axillary LN anlage was surrounded by Lyve-1\(^+\) (Fig. 1C) or Prox1\(^+/\)podoplanin\(^+\) (Fig. 1E) LECs, and central to the cluster of hematopoietic cells, a VEGFR1\(^+/\)VEGFR2\(^+/\)MECA32\(^-\) blood vessel was detected (Fig. 1G; arrowhead). At E16.5, this blood vessel was located distal from the hematopoietic cluster and opposed to the MAdCAM-1\(^+/\)Lyve-1\(^+\) LECs (Fig. 1H; arrowhead). Smaller VEGFR1\(^-/\)VEGFR2\(^+/\)MECA32\(^+\) blood vessels were also located among stromal cells at both developmental stages (Fig. 1G, H).

Next, we determined whether LECs that colocalized with the earliest clusters of LTi cells within the LN anlagen provided some type of inductive signal that is required for the formation of these clusters. To this end and to conclusively address whether LECs/lymph sacs regulate the initiation of mammalian LN development, we took advantage of available Prox1\(^-/-\) mouse embryos \(^5\). Prox1 activity is necessary for the specification of the LEC phenotype in venous endothelial cells located in the embryonic cardinal veins \(^4\). Upon specification of the LEC phenotype by Prox1 and in agreement with Sabin’s original proposal \(^1\), the LEC progenitors leave the cardinal vein, form the primitive lymph sacs and subsequently, the entire lymphatic network \(^4, 5\). In Prox1\(^-/-\) embryos, LEC specification does not take place; therefore, these mutant embryos lack all LEC derivatives such as lymph sacs and lymphatic vasculature \(^4, 5\).

As described above, the presence of LN anlagen in E14.5 Prox1\(^-/-\) embryos was analyzed by screening for clusters of CD45\(^+/\)CD4\(^+\) LTi cells that were in close contact with MAdCAM-1\(^+\) stromal and endothelial cells. Analysis was performed at E14.5, because the LN anlagen can clearly be detected at that stage, and Prox1\(^-/-\) embryos do not survive beyond this stage \(^5\). This analysis revealed that accumulation of LTi cells and MAdCAM-1\(^+\)-expressing stromal cells was not affected in LECs and lymph sac-deficient E14.5 Prox1\(^-/-\) embryos (Fig. 2C, F, I). This result indicated that LECs and/or primitive lymph sacs are not required during the initial step leading to the formation of the LN anlagen. However, the organization of the MAdCAM-1\(^+\) cells appeared to be affected in the mutant LN anlagen. Normally at this stage, MAdCAM-1\(^+\)
Figure 1. Characterization of lymphatic endothelial cells (LECs) in wild-type developing axillary lymph nodes (LNs) at E14.5 (A, C, E, G) and E16.5 (B, D, F, H). (A, B) LNs were identified by combined staining for CD4 (green), which is expressed by LTi cells; Il7r (red), which is expressed by LTi cells and their precursors; and CD45 (blue), which is expressed by all hematopoietic cells. (C-D) Subsequent sections were stained to detect the LEC marker Lyve-1(blue) in combination with the stromal marker MAdCAM-1 (red) and the endothelial cell marker VE-cadherin (green). (E-F) the LEC marker Prox1 (green) in combination with the LN stromal cell marker podoplanin (red) and the vascular endothelial cell marker VEGFR3 (blue), (G-H) and the vascular endothelial cell markers VEGFR2 (green), MECA32 (red), and VEGFR1 (blue). Arrows in (C) and (E) indicate the lining of the lymphatic endothelium. Arrowheads in (G) and (H) indicate blood vessels. Scale bars (A-H), 75 µm.
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Figure 1, continued.
cells encapsulate the hematopoietic cells (arrow in Fig. 2G); instead, in Prox1-null embryos, MA
dCAM-1^+ cells were intermingled with the hematopoietic cells and did not encapsulate the hematopoietic clusters (arrow in Fig. 2I). Also, in contrast to wild-type embryos, E14.5 Prox1^-/- embryos did not appear to contain the inguinal and popliteal LN anlagen. The lack of these anlagen is likely due to a developmental delay of the Prox1-null embryos, because these anlagen form last.

Next, we confirmed that the clusters of CD45^+CD4^+ cells detected in Prox1-null embryos corresponded to LTi cells and therefore truly represent the earliest LN anlagen. To do this, we immunostained sections of E14.5 Prox1-null embryos for RORγ(t), a nuclear orphan receptor required for the generation of LTi cells.

To determine whether reduced levels of Prox1 activity resulting in a defective lymphatic vasculature affect the normal formation of the LN anlagen, we analyzed Prox1-heterozygous mice that exhibited mispatterned and leaky lymphatic vasculature. Expression of CD45, CD4, and Il7r (LTi cells and their precursors); VEGFR1, MEGA32, and VEGFR2 (blood vasculature); and Prox1, podoplanin, and MA
dCAM-1 (LECs and stromal cells, unpublished results) was compared in E14.5 wild-type, Prox1^+/-, and Prox1^-/- embryos. No obvious changes were seen in the morphology or size of the LTi cell clusters (Fig. 3A, B) in the presence of the larger VEGFR1^+/VEGFR2^+/MECA32^- blood vessels (arrows in Fig. 3D, E), in the lining of the lymphatic endothelium (arrows in Fig. 3G, H), or in the presence of the smaller VEGFR1^+/VEGFR2^+/MECA32^- blood vessels (arrowheads in Fig. 3D, E) between wild-type and Prox1-heterozygous littermates. In contrast, the larger VEGFR1^+/VEGFR2^+/MECA32^- blood vessels (Fig. 3F) and the lining of the lymphatic endothelium (Fig. 3I) were not detected in Prox1-null littermates.

Next, we followed the progression of LN formation in adult Prox1-heterozygous mice. To do this, we analyzed the organization of B cells and T cells and the presence of follicular dendritic cells, the T-cell areas harboring fibroblastic reticular cells, and the high endothelial venules and intermediate sinuses in the peripheral and mesenteric LNs. No obvious differences were observed when these results were compared to those from wild-type controls (Fig. S2).

After determining that the lack of LECs and lymph sacs in Prox1-null embryos does not affect the initiation of LN formation and that despite the reduced Prox1 levels in Prox1-heterozygous embryos, LN formation occurs normally, we addressed whether the greatly reduced number of LECs and defective primary lymph sacs would affect the initiation of LN formation or further development of the initial clusters of LTi cells into bona fide LNs. To do this, we took advantage of available Prox1-conditional mutant embryos in which Prox1 activity was removed from venous LEC progenitors. Floxed Prox1 mice were crossed with an available Tie2-Cre transgenic line. Around E10.5, Tie2 is expressed in endothelial cells of the cardinal veins. We have previously shown that upon Prox1 expression,
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Figure 2. Lymph node (LN) anlagen are present in the absence of lymphatic vasculature in Prox1<sup>−/−</sup> embryos. Most LNs are found in E14.5 Prox1<sup>−/−</sup> embryos upon combined staining for MAdCAM-1 (green), CD4 (red), and CD45 (blue). Depicted are mesenteric LNs (A-C), renal LNs (D-F), and axillary LNs (G-I) in wild-type, Prox1<sup>+/−</sup>, and Prox1<sup>−/−</sup> embryos. Arrows indicate MAdCAM-1<sup>+</sup> cells that encapsulate hematopoietic cells (G) or disorganized clusters of hematopoietic and MAdCAM-1<sup>+</sup> cells (I). Scale bars (A-I), 75 µm.
these venous endothelial cells adopt a LEC phenotype and bud from the veins to form the primary lymph sacs \(^4\). Therefore, we used Tie2-Cre to generate Prox1–conditional null embryos in which lymphangiogenesis was severely compromised \(^8\). Although variable, some of these Prox1–conditional mutant embryos contained only a few Prox1-expressing LECs in or around the anterior cardinal vein at around E11.5. At E15.5, they exhibited only some occasional scattered superficial LECs \(^8\). Importantly, no deep lymphatic vasculature was identified in some of the more severely affected mutant embryos \(^8\). In summary, although standard Prox1-null embryos are completely devoid of LECs and therefore of lymph sacs and lymphatic vasculature, severely affected Prox1–conditional mutant embryos exhibit small and morphologically defective lymph sacs (our unpublished observations; \(^8\)).

E17.5 Prox1–conditional mutant embryos were generated by the intercross of Prox1\(^{+/–}\) and Tie-2-Cre/Prox1\(^{flox/+}\) mice \(^8\). As previously indicated \(^8\), only occasional, scattered, superficial Prox1-expressing LECs were present in some of the most severely affected Tie-2-Cre/Prox1\(^{flox/+}\)–mutant embryos. As revealed by immunostaining with antibodies against MAdCAM-1, CD4, and Il7r, all LNs were present in E17.5 Prox1–conditional mutant embryos (data not shown). In the most affected embryo (based on excision efficiency and the limited number of Prox1-expressing LECs), the size of the clustered CD4-expressing LTi cells was greatly reduced (data not shown); in less affected embryos with more Prox1-expressing LECs, normal-appearing clusters of LTi cells that colocalized with MAdCAM-1\(^+\)/Lyve-1\(^+\) cells were observed (Fig. 4B). However, further analysis of the stromal organizer cells within the developing LNs showed reduced expression of MAdCAM-1 and VCAM-1 in the conditional mutant embryos (Fig. 4C, D). These results suggest that LECs and/or the lymphatic vasculature help to position LTi cells in such a way that mesenchymal cells are stimulated to differentiate toward stromal organizers. The reduced number of LECs and lymphatic vessels present in these conditional mutants hamper normal mesenchymal cell differentiation.

Our results conclusively demonstrate that the initial clustering of LTi cells does not require the presence of LECs, lymph sacs, or lymphatic vessels. This result disproves the original model proposed by Sabin a century ago that suggested that lymph sacs ultimately transform into LNs. We also determined that the subsequent organization of the clustered LTi cells was affected in Prox1-null embryos, a result suggesting that LECs and/or lymph sacs are required for the formation of the LN capsule and for the positioning of the hematopoietic cells within the developing LN. The lack of organization observed in LNs of Prox1–conditional mutant embryos could be caused by the absence of enough Ccl21-expressing LECs, because LTi cells responded to Ccl21, and the production of Ccl21 in LECs might further organize the first LTi cells \(^23\).

Additional analyses are necessary to identify the inductive signals involved in attracting and clustering the first LTi cells at the sites where LNs will develop. We have observed that LNs often develop at locations where blood vessels bifurcate; therefore, signals that are required for blood vessel branching might stimulate the initiation of LN formation. Accordingly, the first inductive signals should lead to chemokine expression responsible for attracting the first LTi cells. Which chemokines are instrumental for this process and how they are induced will need further study.
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Figure 3. Lymph node (LN) anlagen develop normally in Prox1-heterozygous and Prox1-nullizygous embryos. To determine whether Prox1 deficiency affects LN development in a dose-dependent manner, we compared the LN anlagen of E14.5 wild-type, Prox1+/–, and Prox1–/– embryos. (A-C) Sections were stained for CD45 (blue), CD4 (green), and II7r (red) to identify clusters of LTi cells, which form the LN anlagen. (D-F) Adjacent sections were stained for VEGFR1 (green), MECA32 (red), and VEGFR2 (blue) to detect endothelial cells and for Prox1 (G, H; green), β-galactosidase (I; β-gal, green), podoplanin (G-I; red) and MAdCAM-1 (blue) to detect lymphatic endothelium and stromal cells within the LN anlagen. LN anlagen of wild-type and Prox1+/– embryos appeared indistinguishable, although the organization of the lymphatic epithelium and blood endothelium within the LN anlagen was disorganized in Prox1–/– embryos. (D-E) Arrows indicate blood vessels, whereas arrowheads indicate small blood vessels. (G-H) Arrows indicate the lining of the lymphatic endothelium. Scale bars (A-H), 75 µm.
Figure 4. Lymph node (LN) anlagen are present in E17.5 Prox1–conditional null embryos (Tie-2-Cre/Prox1^flox/flox). (A, C) Staining of wild-type and Tie-2-Cre/Prox1^flox/flox brachial LNs with antibodies against (A, B) Lyve-1 (green), MAdCAM-1 (red), and CD4 (blue) revealed that Lyve-1^+ lymphatic endothelial cells that coexpress MAdCAM-1 are absent in the LN anlagen of Tie-2-Cre/Prox1^flox/flox embryos. (C, D) Staining of adjacent sections with antibodies against MAdCAM-1 (green), VCAM-1 (red), and podoplanin (blue) indicated that the MAdCAM-1^+/VCAM-1^+ stromal organizer cells, which are abundant in the wild-type LN anlagen (C), are greatly reduced in the Tie-2-Cre/Prox1^flox/flox LN anlagen (D). Scale bars (A-C), 75 µm.
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Figure S1. Lymph node (LN) anlagen contain RORγ(t)-expressing lymphoid tissue inducer (LTi) cells. To confirm that the clustered CD45+/CD4+ cells that colocalize with Madcam-1+ cells are indeed LTi cells, we immunostained sections of (A) E16.5 wild-type, (B) E14.5 wild-type, and (C) E14.5 Prox1-/- embryos with antibodies against RORγ(t) (green), CD4 (red), and CD45 (blue). RORγ(t) expression was detected in CD45+/CD4+ cells of wild-type and Prox1-null embryos, confirming their identity as LTi cells (arrowheads). Scale bars (A-C), 75 µm.
Figure S2. The overall architecture of adult lymph nodes (LNs) from Prox1-heterozygous mice appeared indistinguishable from that of wild-type mice. Peripheral LNs from wild-type or Prox1-heterozygous mice were collected and stained for CD4 (green) and CD8 (red), to identify T cell subsets and B220 (blue) to detect B cells (A, B). Follicular dendritic cells within the B-cell follicle were identified by expression of CD35 (red) and CD16/32 (green), and fibroblastic reticular cells within the T-cell areas were characterized by expression of podoplanin (blue) (C, D). High endothelial venules within the T-cell areas were identified by expression of CD105 (red) and PNAgd, as detected by staining for MECA-79 (green), and were surrounded by Lyve-1+ cells (blue) (E, F). Podoplanin+ (blue) fibroblastic reticular cells within the T-cell areas colocalized with ERTR7+ (green) extracellular matrix components, and some fibroblastic reticular cells expressed Prox-1 (red) (G, H).
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Figure S2, continued.