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

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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 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 initiated normally in E14.5 *Prox1*-null mouse 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.

KEY WORDS: PROX1, Lymphatic endothelial cells, Lymphoid tissue inducer cell, Lymph nodes, Lymph sacs

INTRODUCTION

More than a century ago, Florence Sabin proposed a model for the development of the mammalian lymphatic vasculature (Sabin, 1902). 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 analysis of the expression of *Vegfr3* (*Flt4*), *Vegfc*, *Prox1* and other genes, the activity of which is crucial for the formation of the entire lymphatic vasculature in mammalian embryos (Kaipainen et al., 1995; Kukk et al., 1996; Wigle et al., 2002; Wigle and Oliver, 1999). Expression of *Prox1* is necessary and sufficient for the specification of the LEC phenotype in venous endothelial cells in vivo and in vitro (Hong et al., 2002; Petrova et al., 2002; Wigle et al., 2002). Sabin's original venous model was recently validated using a genetic lineage-tracing approach (Srinivasan et al., 2007).

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 (Sabin, 1909). A century later, this dogma has remained unchallenged despite the recent availability of appropriate molecular markers and mouse models, and it is still 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 homozygous null, hemizygous or conditionally removed from venous LEC progenitors. In these mutant mice, lymph sacs are either absent or defective, providing an ideal system in which to evaluate whether lymph sacs are required for LN formation.

We conclude that primitive lymph sacs are not necessary for the initial formation of the mammalian LN anlagen. However, their 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) and lymphotoxin-alpha-deficient (*Lta*$^{-/-}$) mice were purchased from Charles River (Maastricht, The Netherlands). The generation of *Prox1*$^{+/-}$, *Prox1*$^{-/-}$, *Tie2-Cre* and *Prox1*$^{flox/flox}$ mice was reported previously (Wigle and Oliver, 1999; Kisanuki et al., 2001; Harvey et al., 2005). 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 sacrificed at different time points, and embryos harvested and prepared for sectioning by embedding and freezing in OCT (Sakura Finetek Europe, Zoeterwoude, The Netherlands).

Immunofluorescence
Following cryosectioning (7 μm) of the embryos, sections were fixed in dehydrated acetone for 2 minutes and then air dried for 15 minutes. Endogenous avidin was blocked with an avidin-biotin block (Vector Laboratories, Burlingame, CA). Sections were then preincubated in PBS supplemented with 5% (v/v) mouse serum for 10 minutes. Incubation with the primary antibody for 45 minutes was followed by incubation with Alexa-Fluor-labeled conjugate (Invitrogen, Breda, The Netherlands) for 30 minutes. All incubations were carried out at room temperature. Sections were counterstained with Hoechst 33342 (Invitrogen) for 10 minutes and analyzed on a Leica TCS SP2 confocal laser-scanning microscope (Leica Microsystems, Rijswijk, The Netherlands).

Antibodies
The antibodies GK1.5 (anti-CD4), MECA-367 [anti-mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1)], MP33 (anti-CD45), 8.1.1 (anti-podoplanin) and ERTR7 (which recognizes extracellular matrix component secreted by fibroblastic reticular cells) were affinity

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purified from the supernatants of hybridoma cell cultures using protein G-Sepharose (Pharmacia, Uppsala, Sweden). The antibodies were biotinylated or labeled with Alexa-Fluor 488, 546 or 633 (Invitrogen). The antibodies A7R34 (anti-IL7Rα; eBioscience, San Diego, CA), 429 (anti-VCAM1; eBioscience), Avas12a1 (anti-VEGFR2; eBioscience), anti-MECA32 (pan-endothelial cell marker; BD Biosciences, Erembodegem, Belgium), anti-LYVE1 (Millipore, Billerica, MA), 11D4.1 [anti-vascular endothelial (VE)-cadherin; BD Biosciences], anti-VEGFR1 (anti-FLT1; Neomarkers, Fremont, CA), AFL4 (anti-VEGFR3; eBioscience), anti-PROX1 (ReliaTech, Braunschweig, Germany), anti-RORγt (kindly provided by D. Littman) (Sun et al., 2000) and anti-β-galactosidase (MP Biomedicals, Aurora, OH) were used biotinylated or unconjugated and visualized with Alexa-Fluor 488, 546 or 633-conjugated streptavidin, anti-rat or anti-rabbit IgG, or anti-Armenian hamster-Cy3, as appropriate.

RESULTS AND DISCUSSION
Identification of LECs in the LN anlagen
To determine the origin of mammalian LNs, we first assessed the contribution of LECs to the developing LNs of E14.5 and E16.5 wild-type mouse embryos. It is well established that early during LN organogenesis and upon their interaction with lymphotoxin-expressing hematopoietic lymphoid tissue inducer (LTi) cells, lymphotoxin-beta receptor (LTβR)-expressing mesenchymal cells differentiate into specialized stromal organizer cells (Mebius, 2003; Vondenhoff et al., 2007). However, before this LTβR-dependent process initiates, some type of signal induces the accumulation of LTi cells and stromal cells (Eberl et al., 2004; White et al., 2007; Yoshida et al., 2002). Until now, the inductive signals from the primitive 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 (Mebius et al., 1996), IL7Rα, which is expressed by LTi cells and their precursors (Cupedo et al., 2004; Yoshida et al., 2002) and CD45 (PTPRC – Mouse Genome Informatics), which is expressed by all hematopoietic cells. This analysis revealed that ~50% of the CD45^{+} cells in the anlagen corresponded to LTi cells, as indicated by their expression of CD4 and IL7Rα 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 MAdCAM1, VE-cadherin (cadherin 5), VEGFR1 (FLT1), VEGFR2 (KDR), MECA32 (PLVAP) and with the LEC markers LYVE1, podoplanin, PROX1 and VEGFR3 (FLT4) (Banerji et al., 1999; Breier et al., 1996; Breiteneder-Geleff et al., 1999; Wigle and Oliver, 1999; Cupedo et al., 2004; Kaipainen et al., 1995; Mebius et al., 1996; Hallmann et al., 1995). At E14.5, the axillary LN anlage was surrounded by LYVE1^{+} (Fig. 1C) or PROX1^{+} and 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 MAdCAM1^{+} LYVE1^{+} LECs (Fig. 1H, arrowhead). Smaller VEGFR1^{–} VEGFR2^{+} MECA32^{+} blood vessels were also located among stromal cells at both developmental stages (Fig. 1G,H).
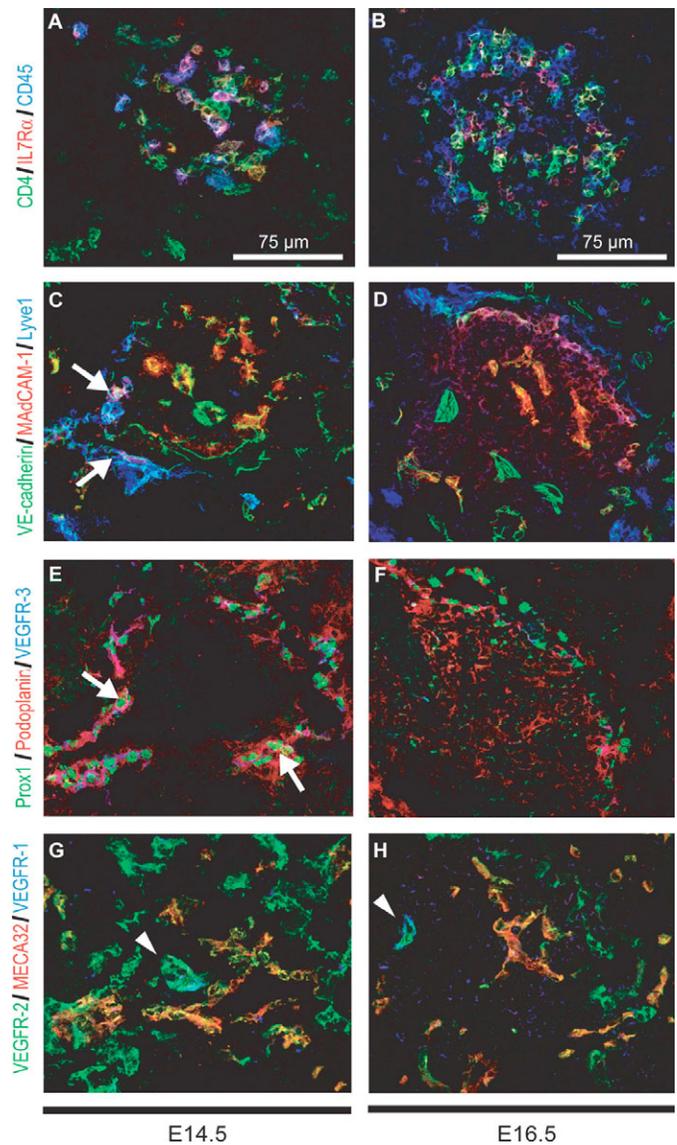

Fig. 1. Characterization of lymphatic endothelial cells in wild-type developing axillary lymph nodes. Lymph nodes (LNs) were examined at E14.5 (A,C,E,G) and E16.5 (B,D,F,H). (**A,B**) LNs were identified by combined staining for CD4 (green), which is expressed by LTi cells, IL7Rα (red), which is expressed by LTi cells and their precursors, and CD45 (blue), which is expressed by all hematopoietic cells. (**C-H**) Subsequent sections were stained to detect (C,D) the lymphatic endothelial cell (LEC) marker LYVE1(blue) in combination with the stromal marker MAdCAM1 (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), and (G,H) 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: 75 µm in A-H.

Lymph sacs are not required to initiate LN formation
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.
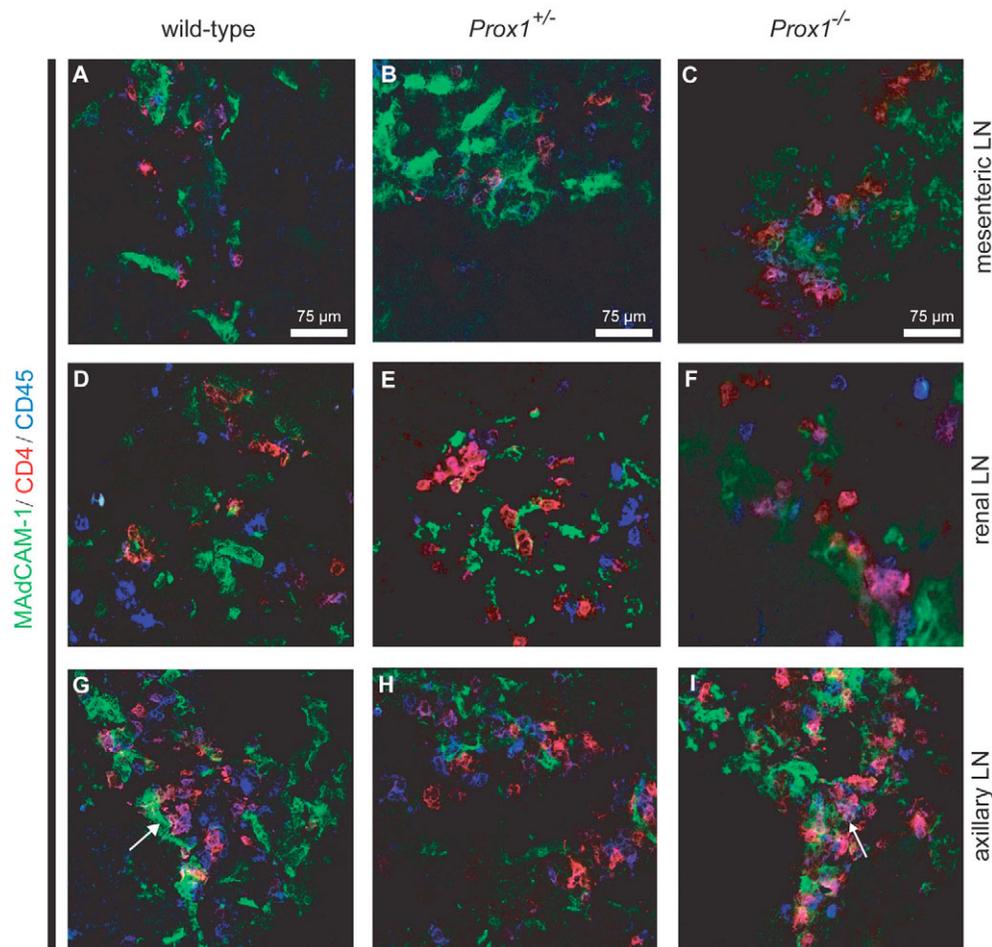
Fig. 2. Lymph node anlagen are present in the absence of lymphatic vasculature in *Prox1⁻/⁻* mouse embryos. Combined staining for MAdCAM1 (green), CD4 (red) and CD45 (blue) indicates that most LN anlagen are present in E14.5 *Prox1⁻/⁻* embryos. Shown are (**A-C**) mesenteric LNs, (**D-F**) renal LNs and (**G-I**) axillary LNs in wild-type, *Prox1⁺/⁻* and *Prox1⁻/⁻* embryos. Arrows indicate MAdCAM1⁺ cells that encapsulate hematopoietic cells (G) or disorganized clusters of hematopoietic and MAdCAM1⁺ cells (I). Scale bars: 75 μm in A-I.

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 (Wigle and Oliver, 1999). *Prox1* activity is necessary for the specification of the LEC phenotype in venous endothelial cells located in the embryonic cardinal veins (Wigle et al., 2002). Upon specification of the LEC phenotype by PROX1 and in agreement with Sabin's original proposal (Sabin, 1902), the LEC progenitors leave the cardinal vein, form the primitive lymph sacs and, subsequently, the entire lymphatic network (Wigle and Oliver, 1999; Wigle et al., 2002). In *Prox1*-null embryos, LEC specification does not take place; therefore, these mutant embryos lack all LEC derivatives, such as lymph sacs and lymphatic vasculature (Wigle and Oliver, 1999; Wigle et al., 2002).

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 MAdCAM1⁺ stromal and endothelial cells. Analysis was performed at E14.5, when LN anlagen can clearly be detected, as *Prox1⁻/⁻* embryos do not survive beyond this stage (Wigle and Oliver, 1999). This analysis revealed that the accumulation of LTi cells and MAdCAM1-expressing stromal cells was not affected in the LEC- and lymph sac-deficient E14.5 *Prox1*-null 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 MAdCAM1⁺ cells appeared to be affected in the mutant LN anlagen. Normally, at this stage, MAdCAM1⁺ cells

encapsulate the hematopoietic cells (Fig. 2G, arrow); instead, in *Prox1*-null embryos, MAdCAM1⁺ cells were intermingled with the hematopoietic cells and did not encapsulate the hematopoietic clusters (Fig. 2I, arrow). Also, in contrast to wild-type embryos, E14.5 *Prox1⁻/⁻* embryos did not appear to contain the inguinal and popliteal LN anlagen. As these anlagen normally form last, their absence is likely to be due to a developmental delay of the *Prox1*-null embryos.

Next, we confirmed that the clusters of CD45⁺CD4⁺ cells detected in *Prox1*-null embryos correspond to LTi cells and therefore truly represent the earliest LN anlagen. To do this, we immunostained sections of E14.5 *Prox1*-null embryos for RORγt (RORC), a nuclear orphan receptor required for the generation of LTi cells (Eberl et al., 2004). The clustered CD45⁺CD4⁺ hematopoietic cells within the early LN anlagen expressed RORγt, thereby confirming their LTi cell identity (see Fig. S1A-C in the supplementary material). This initial analysis conclusively demonstrated that, contrary to the accepted dogma, the initiation of mammalian LN formation does not require lymph sacs.

Defective lymphatic vasculature does not affect LN anlagen formation

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 (Harvey et al., 2005). Expression of CD45, CD4 and IL7Rα (LTi cells and their precursors),
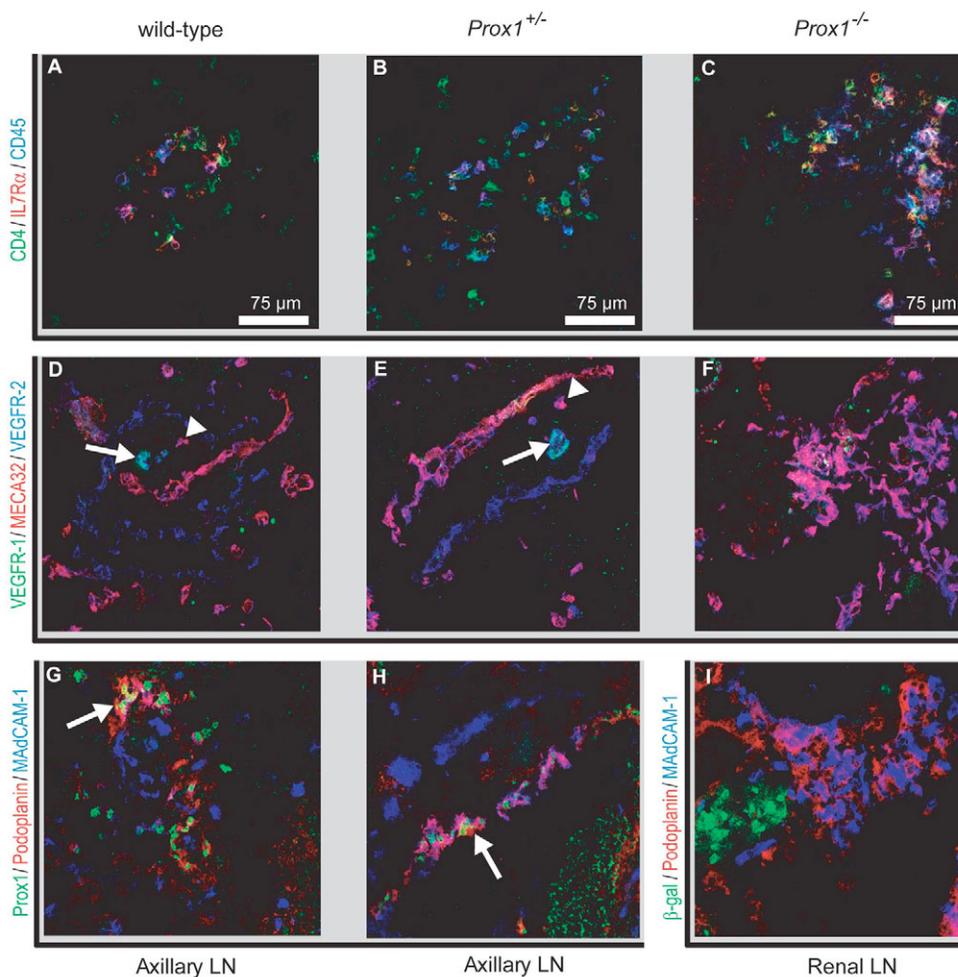
Fig. 3. Lymph node anlagen develop normally in *Prox1*-heterozygous and *Prox1*-null mouse embryos. To determine whether PROX1 deficiency affects LN development in a dose-dependent manner, we compared the LN anlagen of E14.5 wild-type, *Prox1*^{+/–} and *Prox1*^{–/–} embryos. (**A-C**) Sections were stained for CD45 (blue), CD4 (green) and IL7Rα (red) to identify clusters of LTi cells, which form the LN anlagen. (**D-I**) Adjacent sections were stained for (D-F) VEGFR1 (green), MECA32 (red) and VEGFR2 (blue) to detect endothelial cells, and for PROX1 (G,H, green), β-galactosidase (β-gal) (I, green), podoplanin (G-I, red) and MAdCAM1 (G-I, 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. Arrows in D and E indicate blood vessels, and arrowheads indicate small blood vessels. Arrows in G and H indicate the lining of the lymphatic endothelium. Scale bars: 75 μm in A-I.

VEGFR1, MECA32 and VEGFR2 (blood vasculature) and of PROX1, podoplanin and MAdCAM1 (LECs and stromal cells; our unpublished results) (Cupedo et al., 2004; Mebius et al., 1996) 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 occurrence of the larger VEGFR1^{+} VEGFR2^{+} MECA32^{–} blood vessels (Fig. 3D,E, arrows), in the lining of the lymphatic endothelium (Fig. 3G,H, arrows), or in the occurrence of the smaller VEGFR1^{–} VEGFR2^{+} MECA32^{+} blood vessels (Fig. 3D,E, arrowheads) between wild-type and *Prox1*-heterozygous littermates. By 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, of the T-cell areas harboring fibroblastic reticular cells, and of the high endothelial venules and intermediate sinuses in the peripheral and mesenteric LNs. No obvious differences were observed to wild-type controls (see Fig. S2 in the supplementary material).

Progression of LN formation is affected in Prox1 conditional mutant mice

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 a greatly reduced number of LECs and the defective primary lymph sacs would affect the initiation of LN formation or the 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 (Harvey et al., 2005) in which *Prox1* activity is removed from venous LEC progenitors. Floxed *Prox1* mice (Harvey et al., 2005) were crossed with a *Tie2-Cre* transgenic line. Around E10.5, Tie2 (Tek) is expressed in endothelial cells of the cardinal veins (Kisanuki et al., 2001; Sato et al., 1993; Harvey et al., 2005; Srinivasan et al., 2007). We have previously shown that upon PROX1 expression, these venous endothelial cells adopt a LEC phenotype and bud from the veins to form the primary lymph sacs (Wigle et al., 2002). Therefore, we used *Tie2-Cre* to generate *Prox1* conditional-null embryos in which lymphangiogenesis is severely compromised (Srinivasan et al., 2007). Although variable, some of these *Prox1* conditional mutant embryos contain only a few PROX1-expressing LECs in or around the anterior cardinal vein at ~E11.5. At E15.5, they exhibit only some occasional, scattered superficial LECs (Srinivasan et al., 2007). Importantly, some of the more severely affected mutant embryos exhibit no deep lymphatic vasculature (Srinivasan et al., 2007). 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) (Srinivasan et al., 2007).
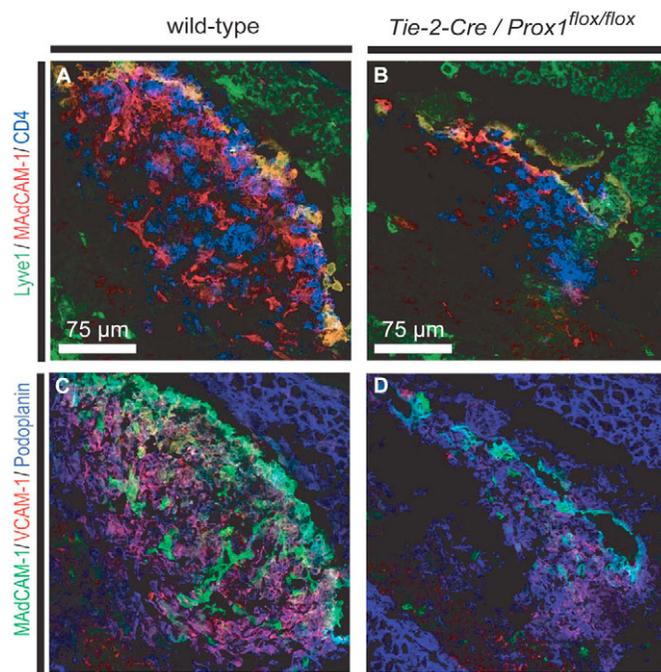
**Fig. 4. Lymph node anlagen are present in E17.5 *Prox1*
conditional-null mouse embryos.** (**A**,**B**) Staining of wild-type and
conditional-null (*Tie2-Cre/Prox1$^{flox/flox}$*) brachial LNs with antibodies
against LYVE1 (green), MAdCAM1 (red) and CD4 (blue) revealed that
LYVE1$^+$ LECs that coexpress MAdCAM1 are absent from the LN anlagen
of *Tie2-Cre/Prox1$^{flox/flox}$* embryos. (**C**,**D**) Staining of adjacent sections
with antibodies against MAdCAM1 (green), VCAM1 (red) and
podoplanin (blue) indicated that the MAdCAM1$^+$ VCAM1$^+$ stromal
organizer cells, which are abundant in the wild-type LN anlagen (C), are
greatly reduced in the *Tie2-Cre/Prox1$^{flox/flox}$* LN anlagen (D). Scale bars:
75 μm in A-D.

E17.5 *Prox1* conditional mutant embryos were generated by the
intercross of *Prox1$^{+/-}$* and *Tie2-Cre/Prox1$^{flox/+}$* mice (Srinivasan et
al., 2007). As previously indicated (Srinivasan et al., 2007), only
occasional, scattered superficial PROX1-expressing LECs were
present in some of the most severely affected *Tie2-Cre/Prox1$^{flox/-}$*
mutant embryos. As revealed by immunostaining with antibodies
against MAdCAM1, CD4 and IL7Rα, all LNs were present in E17.5
Prox1 conditional mutant embryos (data not shown). In the most
severely affected embryo (based on excision efficiency and the
limited number of PROX1-expressing LECs), the size of the CD4-
expressing LTi cell clusters was greatly reduced (data not shown).
In less severely affected embryos with more PROX1-expressing
LECs, LTi cell clusters of normal appearance and that colocalized
with MAdCAM1$^+$ LYVE1$^+$ cells were observed (Fig. 4B). However,
further analysis of the stromal organizer cells within the developing
LNs showed reduced expression of MAdCAM1 and VCAM1 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 indicating 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 which suggests 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 a deficiency in CCL21-expressing LECs, as LTi
cells respond to CCL21 and the production of CCL21 in LECs might
further organize the first LTi cells (Honda et al., 2001).

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 that is 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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