Conclusions—These results reveal an important role of podoplanin in lymphatic vessels in preventing postnatal blood filling of the lymphatic vascular system and in contributing to efficient dendritic cell migration to the lymph nodes. (Arterioscler Thromb Vasc Biol. 2017;37:108-117. DOI: 10.1161/ATVBAHA.116.308020.)

Key Words: endothelium, lymphatic ▪ fluorescein-5-isothiocyanate ▪ lymph nodes ▪ models, animal ▪ thoracic duct

Postnatal Deletion of Podoplanin in Lymphatic Endothelium Results in Blood Filling of the Lymphatic System and Impairs Dendritic Cell Migration to Lymph Nodes

Roberta Bianchi, Erica Russo, Samia B. Bachmann, Steven T. Proulx, Marko Sesartic, Nora Smaadahl, Steve P. Watson, Christopher D. Buckley, Cornelia Halin, Michael Detmar

Objective—The lymphatic vascular system exerts major physiological functions in the transport of interstitial fluid from peripheral tissues back to the blood circulation and in the trafficking of immune cells to lymph nodes. Previous studies in global constitutive knockout mice for the lymphatic transmembrane molecule podoplanin reported perinatal lethality and a complex phenotype with lung abnormalities, cardiac defects, lymphedema, blood-filled lymphatic vessels, and lack of lymph node organization, reflecting the importance of podoplanin expression not only by the lymphatic endothelium but also by a variety of nonendothelial cell types. Therefore, we aimed to dissect the specific role of podoplanin expressed by adult lymphatic vessels.

Approach and Results—We generated an inducible, lymphatic-specific podoplanin knockout mouse model (PdpnΔLEC) and induced gene deletion postnatally. PdpnΔLEC mice were viable, and their lymphatic vessels appeared morphologically normal with unaltered fluid drainage function. Intriguingly, PdpnΔLEC mice had blood-filled lymph nodes and vessels, most frequently in the neck and axillary region, and displayed a blood-filled thoracic duct, suggestive of retrograde filling of blood from the blood circulation into the lymphatic system. Histological and fluorescence-activated cell sorter analyses revealed normal lymph node organization with the presence of erythrocytes within lymph node lymphatic vessels but not surrounding high endothelial venules. Moreover, fluorescein isothiocyanate painting experiments revealed reduced dendritic cell migration to lymph nodes in PdpnΔLEC mice.

Conclusions—These results reveal an important role of podoplanin expressed by lymphatic vessels in preventing postnatal blood filling of the lymphatic vascular system and in contributing to efficient dendritic cell migration to the lymph nodes. (Arterioscler Thromb Vasc Biol. 2017;37:108-117. DOI: 10.1161/ATVBAHA.116.308020.)

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In podoplanin-deficient embryos,25–27 and deletion of podoplanin including hypoplasia of the myocardium, were described in Pdpn knockout mice display congenital lymphedema, impaired lymphatic function, dilation and mispatterning of the intestinal and cutaneous lymphatic vasculature, and blood-filled LVs.20–22 The latter phenotype has been attributed to the interaction, at midgestation, of podoplanin expressed by LECs with CLEC-2 expressing platelets, inducing platelet activation and formation of thrombi that mediate the proper separation of the nascent lymphatic system from the cardinal vein.22,23 These mice also lack most of the LNs that are replaced by blood-filled, poorly organized remnants.24 In addition, several cardiac defects, including hypoplasia of the myocardium, were described in podoplanin-deficient embryos,25–27 and deletion of podoplanin at the 2-cell stage causes cerebral hemorrhaging in embryos.28

The broad expression of podoplanin, together with the lethal phenotype observed in global podoplanin-deficient mice, suggests an important role for podoplanin in diverse cell types and in the ontogeny of different organs. It has, however, not been possible to date to dissect its direct function specifically in the lymphatic endothelium at late postnatal or adult stages. To specifically investigate the function of podoplanin in the postnatal lymphatic vasculature, we developed an inducible, lymphatic-specific podoplanin knockout mouse by crossing Pdpn mice28 and Prox1-Cre-ERT2 mice29 to obtain the Pdpn ΔLEC line (Figure 1A). Postnatal deletion of the podoplanin gene was induced by daily tamoxifen administrations from postnatal day (P) 1 to P3 (Figure 1A). Adult PdpnΔLEC mice showed efficient podoplanin downregulation in dermal LVs, as assessed by whole-mount immunofluorescence stainings for podoplanin and the lymphatic marker LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1; Figure 1B; Figure ID in the online-only Data Supplement), and by fluorescence-activated cell sorter analysis (Figure 1C). PdpnΔLEC mice were monitored

### Results

#### Generation of a Conditional, Lymphatic-Specific Podoplanin Knockout Mouse Model

Because global constitutive podoplanin knockout mice display a lethal phenotype on most genetic backgrounds, at least in part caused by a failure to inflate their lungs,18 we generated mice with postnatal deletion of podoplanin specifically in the lymphatic vasculature. To this aim, PdpnΔ mice were crossed with Prox1-Cre-ERT2 mice,28 which express Cre recombinase under control of the lymphatic specification gene Prox11 in response to tamoxifen administration, obtaining the PdpnΔLEC line (Figure 1A).

Postnatal deletion of the podoplanin gene was induced by daily tamoxifen administrations from postnatal day (P) 1 to P3 (Figure 1A). Adult PdpnΔLEC mice showed efficient podoplanin downregulation in dermal LVs, as assessed by whole-mount immunofluorescence stainings for podoplanin and the lymphatic marker LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1; Figure 1B; Figure ID in the online-only Data Supplement), and by fluorescence-activated cell sorter analysis (Figure 1C). PdpnΔLEC mice were monitored
from birth to at least 23 weeks of age; they were not visually distinguishable from wild-type littermates, and their weight was normal (data not shown).

Postnatal Deletion of Podoplanin Does Not Interfere With Lymphatic Patterning

At P7, Pdpn\textsuperscript{\textDelta\textsubscript{LEC}} pups appeared normal, and no difference in weight was observed (Figure 2A). Podoplanin was efficiently downregulated at this time point, as assessed by podoplanin whole-mount stainings of abdominal skin, diaphragm, and mesentery (Figure 1A through 1C in the online-only Data Supplement). LV morphology was analyzed by staining whole-mount preparations of abdominal skin (Figure 2B), tail skin (Figure 2C), and diaphragm (Figure 2D) for LYVE-1. Morphometric analyses did not reveal any significant differences in the LYVE-1\textsuperscript{+} area, number of branch points, number of ring structures, LV diameter, total vessel length, and average branch length (Figure 2B through 2D).

Adult LVs Are Morphologically and Functionally Normal After Postnatal Deletion of Podoplanin

Next, we investigated the morphology and functionality of adult LVs lacking podoplanin. To this purpose, ear whole-mounts of 11-week-old mice were stained for LYVE-1. LV morphology appeared normal in adult Pdpn\textsuperscript{\textDelta\textsubscript{LEC}} animals, as no major differences were found in the tissue area covered by LVs, the number of LV branches, the total vessel length, and the average vessel diameter (Figure 3A). As a measure of lymphatic function, we injected the lymphatic-specific tracer P20D800\textsuperscript{31,32} intradermally into the ear and monitored its clearance by LVs over time. As quantified by half-life and K rate of the tracer decay, no difference was observed in

**Figure 2.** Postnatal deletion of podoplanin does not interfere with lymphatic patterning. A, Comparable body weights of Pdpn\textsuperscript{\textDelta\textsubscript{LEC}} mice and wild-type littermates at P7. Unpaired Student t test, \textit{P}>0.05. B, Confocal z-stack images of representative whole-mounts of abdominal skin samples stained for LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1; green). Quantification of the LYVE-1\textsuperscript{+} area and number of branch points per image showed no difference between genotypes. Mann–Whitney \textit{U} test, \textit{P}>0.05. C, Confocal z-stack images of representative tail skin whole-mount samples stained for LYVE-1. Quantification of the LYVE-1\textsuperscript{+} area and the number of ring-like structures per image revealed no difference between genotypes. Mann–Whitney \textit{U} test, \textit{P}>0.05. D, Confocal z-stack images of representative diaphragm segments stained for LYVE-1. Quantification of the LYVE-1\textsuperscript{+} area, number of branch points, total vessel length, average vessel diameter, and average branch length per diaphragmatic segment showed no difference between genotypes. Mann–Whitney \textit{U} test, \textit{P}>0.05. Four to 5 animals per genotype from 4 independent litters were analyzed. Scale bars, 100 \textmu m. LV indicates lymphatic vessel.
lymphatic clearance between Pdpn\textsuperscript{ALEC} mice and wild-type littermates (Figure 3B).

### Postnatal Deletion of Podoplanin Results in Blood-Filled LVs and LNs

The LNs of adult Pdpn\textsuperscript{ALEC} mice often displayed a bloody appearance (Figure 4A and 4C; Figure IIA and IIB in the online-only Data Supplement). To determine when this phenotype arose, we analyzed animals at different ages, namely, P7, P21, and adults. As shown by the phenotype frequencies in different anatomic locations (Figure 4B), at P7, the LNs were never blood-filled and we only observed blood in the TD of 1 of 9 mice analyzed. At weaning age (P21), the phenotype was apparent and, as in adult mice, the LNs located in the neck (auricular and mandibular) and axilla (axillary and brachial) showed the phenotype with higher frequency than the inguinal LNs and flank lymphatic collectors (Figure 4C). The mesenteric LNs (Figure IIB in the online-only Data Supplement) and intestinal LVs (Figure 4D) were blood-filled only in adult Pdpn\textsuperscript{ALEC} mice and were not affected at P21 or P7.

Fluorescence-activated cell sorter analysis revealed an increase in the frequency of red blood cells (identified as CD45\textsuperscript{+} Ter119\textsuperscript{+} events) in auricular LNs harvested from adult Pdpn\textsuperscript{ALEC} mice (7.7±2.17% of cells) when compared with wild-type mice (1.3±0.53%), confirming the presence of blood in the LNs (Figure 4E).

To determine the origin of bleeding, we performed immunofluorescence analyses for red blood cells (Ter119\textsuperscript{+}), LYVE-1, and the pan-endothelial marker CD31 or VE-cadherin on frozen LN sections (Figure 4F; Figure IIC and IID in the online-only Data Supplement). Red blood cells were detected inside not only CD31\textsuperscript{+}/LYVE-1 HEVs (Figure 4F, arrowheads) but also LVs in Pdpn\textsuperscript{ALEC} mice (Figure 4F, arrows and high-power inset; Figure IID in the online-only Data Supplement).

Importantly, no red blood cells were observed in the proximity of HEVs, suggesting that extravasation of erythrocytes from these vessels did not occur. Because FRCs lining HEVs retained podoplanin expression in Pdpn\textsuperscript{ALEC} mice (Figure III in the online-only Data Supplement, arrowheads), the presence of blood in the LNs was not because of compromised HEV integrity, but because of blood filling of the lymphatic vasculature.

We also analyzed the presence of blood in the TD of Pdpn\textsuperscript{ALEC} mice, which was identified by fluorescent lymphatic tracing after intradermal injection of the lymphatic tracer P20D800\textsuperscript{J1} in both hind paws. Blood was visible by white-light analysis in the TD in 36.4% (4 of 11) of Pdpn\textsuperscript{ALEC} mice (Figure 4G) but not in wild-type mice (n=7). In the majority of Pdpn\textsuperscript{ALEC} mice (3 of 4), the blood-filled TD was massively dilated (Figure 4G). Importantly, only mice that showed blood in the TD also had blood-filled mesenteric LNs (Figure IB in the online-only Data Supplement). Taken together, these data suggest that the lack of podoplanin on LECs results in retrograde flow of blood from the veins into the lymphatic system.

To investigate whether podoplanin is required throughout life to prevent blood filling of the lymphatic system, we induced gene deletion in adult mice by 5 daily tamoxifen injections and analyzed the phenotype 5 days after the last tamoxifen injection (Figure IVA in the online-only Data Supplement). With this regimen, podoplanin expression was strongly reduced even though gene deletion was not complete, as a low level of podoplanin expression was detected in some dermal LVs by whole-mount staining (Figure IVB in the online-only Data Supplement). We did not observe any blood in the LNs, but in one-third of the mice blood filled the TD (Figure IVC in the online-only Data Supplement). These data indicate that podoplanin is required throughout life to keep the blood from entering the lymphatic system.
Postnatal Deletion of Podoplanin Does Not Compromise LN Organization

Global podoplanin knockout mice have poorly organized LNs. To assess the contribution of LV podoplanin to LN organization, we analyzed the morphology of PdpnΔLEC LNs in more detail. Histological analyses revealed normal B-cell follicle organization in PdpnΔLEC LNs, as compared with wild-type controls (Figure 5A). Ear draining LNs of PdpnΔLEC mice were heavier, whereas the total leukocyte number measured after RBC lysis, the proportions of T cells, and the absolute number of migratory DCs did not differ between the genotypes (Figure 5B through 5F). We also analyzed HEVs by immunostaining for the differentiation marker MECA79 and observed a normal HEV morphology in LNs of PdpnΔLEC mice (Figure 5G). We found heavier LNs in PdpnΔLEC mice than in wild-type controls (Figure 5B). To assess the relative contribution of the presence of RBC and of the accumulation of fluid to the increased weight observed, we analyzed the wet weight and dry weight of mandibular LNs and calculated the LN fluid content. Both the wet and dry LN weights were increased in PdpnΔLEC mice when compared with wild-type controls, and the LN fluid content did not differ between the genotypes (Figure 5H). These data, together with the results on total LN cellularity (Figure 5C), indicate that the presence of RBC is responsible for the increased LN weight observed in PdpnΔLEC mice.
Postnatal Deletion of Podoplanin Impairs DC Migration

It has been suggested that CLEC-2 deficiency hampers DC migration to the LN. To investigate whether the CLEC-2 binding partner podoplanin on LVs contributes to efficient DC trafficking to the LN, we applied FITC solution to the ears of mice and analyzed its uptake by DCs and their migration to the draining auricular LNs after 18 hours (Figure 6A). We found reduced proportions of FITC+ DCs (gated as MHCII+ CD11c+ cells) in the LNs of PdpnLEC mice when compared with wild-type controls (Figure 6B). This reduction was a consequence of impaired DC migration, as there were no differences in the proportion of CD11b+ CD11c+ dermal DCs in the ear skin of PdpnLEC mice (Figure 6C). Fluorescence-activated cell sorter analysis confirmed that LN DCs expressed CLEC-2 and that PdpnLEC mice retained normal CLEC-2 expression (Figure 6D).

Discussion

Podoplanin is not only a marker of lymphatic endothelium but also expressed by other cell types such as LN FRCs and alveolar type I cells in the lung. Thus, global constitutive podoplanin-deficient mice display a complex phenotype characterized by death at birth because of lung abnormalities, heart defects, lack of most LNs, edema, and blood-filled LVs. Here, we generated and characterized a mouse model for the postnatal deletion of podoplanin specifically in LVs.

Podoplanin is the only described endogenous ligand of CLEC-2, and this biochemical interaction has been investigated in detail. The extracellular portion of podoplanin contains conserved domains required for CLEC-2 engagement, which in platelets results in the phosphorylation of the CLEC-2 cytoplasmic tail, activation of the Syk tyrosine kinase, and consequent downstream signaling via PLCγ2 (phospholipase C gamma 2), leading to platelet activation. It has been proposed that during embryonic development, podoplanin/CLEC-2–induced platelet activation prevents the blood filling of the budding lymphatic system from the cardinal vein. Indeed, mice lacking podoplanin, CLEC-2, or downstream signaling molecules, such as Syk and SLP-76, all display a similar blood-filled lymphatic phenotype.

We induced podoplanin deletion during early postnatal days (P1 to P3); since at this time point, the lymphatic system has already separated from the cardinal vein, and lymphatic plexuses are actively maturing in different organs. We did not observe lethality or gross developmental defects up to 23 weeks of age in PdpnLEC animals, suggesting that postnatal deletion of podoplanin in lymphatic endothelium is compatible with life.

Analysis of P7 pups and adult mice revealed morphologically normal and functional LVs in PdpnLEC mice, suggesting that podoplanin does not play a major role in the postnatal maturation of LVs or in the maintenance of adult LV morphology and drainage function. Previous analyses of global constitutive podoplanin-deficient mice showed enlarged and disorganized LVs, as well as edema and impaired lymphatic function. In addition, blocking of podoplanin function by administration of podoplanin-Fc during late embryonic development resulted in a less complex diaphragmatic lymphatic network in pups. Thus, it is likely that, early in development,
podoplanin functionally contributes not only to the separation of the blood vascular and the lymphatic system but also to LV patterning. However, our data indicate that after birth, podoplanin is dispensable for proper LV maintenance and drainage function. In global podoplanin-deficient mice, where podoplanin is also deleted from LN FRCs, lymphatic function is likely impaired by the lack of LNs.24 In contrast, in Pdpn\textsuperscript{\textDelta LEC} mice, LNs are present and are normally organized, indicating that podoplanin expressed by FRCs and not by LECs is responsible for the lack of LN organization observed in global constitutive podoplanin-deficient mice.

The most striking phenotype observed in Pdpn\textsuperscript{\textDelta LEC} mice was the presence of blood-filled LN LVs especially in the neck and axilla (95.5% and 68.2% of adult Pdpn\textsuperscript{\textDelta LEC} mice, respectively) and, less frequently, flank collecting LVs (7.6% of adult Pdpn\textsuperscript{\textDelta LEC} mice) and mesenteric LNs and intestinal LVs (31.8% and 10.6% of adult Pdpn\textsuperscript{\textDelta LEC} mice, respectively). Moreover, blood was present in the TD of 36.4% of adult Pdpn\textsuperscript{\textDelta LEC} mice analyzed. Interestingly, LN LVs were never blood-filled in P7 Pdpn\textsuperscript{\textDelta LEC} mice (ie, 5 days after the last tamoxifen injection) and only 11% of mice showed a blood-filled TD at this time point. At weaning age (P21), the frequency of the phenotype was similar to adult mice, with the exception of the mesenteric LNs and LVs, which were never blood-filled at this time point. These data suggest that, after podoplanin deletion, the blood takes a few weeks to reach the LN LVs and more distal sites, such as the mesentery. Accordingly, when gene deletion was induced in adult mice, LN LVs were not blood-filled 5 days after the last tamoxifen injection, but blood was detected in the TD of 1/3 of the mice, similar to what we observed at P7 after tamoxifen injections at P1 to P3.

Moreover, the incomplete penetrance of this phenotype might be because of partial podoplanin deletion, which can occur when Cre recombination is induced after embryonic development.42 Efficient gene deletion relies on sufficient tamoxifen bioavailability in a given cell/organ, Cre promoter activity, and expression levels of the target gene and local chromatin conformation at the loxP sites, and it is possible that these parameters vary between different cells.42–44

Taken together, these data allowed us to propose a potential mechanism of the blood filling of LVs in Pdpn\textsuperscript{\textDelta LEC} mice. The observation that the LNs located in the neck and axilla, which are anatomically closer to the lymphovenous valves, were filled by blood with the highest frequency and that the phenotype was more pronounced in adult mice than at P7 suggests that LV expression of podoplanin regulates blood–lymph separation in adults and that loss of podoplanin likely compromises platelet aggregation in the proximity of the lymphovenous valve, allowing filling of blood into the lymphatic system (Figure V in the online-only Data Supplement). Our observations confirm and extend a recent study that revealed an important function of platelet CLEC-2 in lymphovenous

Figure 6. Postnatal deletion of podoplanin impairs dendritic cell (DC) migration to lymph nodes (LNs). A, Schematic representation of the fluorescein isothiocyanate (FITC) painting experiment: FITC solution was applied to the ears, and the draining auricular LNs (auLNs) were harvested and analyzed by fluorescence-activated cell sorter (FACS) after 18 h. B, Representative FACS dot plots (gated on MHCII\textsuperscript{+} events) showing MHCII\textsuperscript{+} CD11c\textsuperscript{+} FITC\textsuperscript{+} DCs in wild-type and Pdpn\textsuperscript{\textDelta LEC} mice 18 h after FITC application. Mann–Whitney U test, *P<0.05. Pooled data from 2 independent experiments analyzing a total of 7 mice per genotype are shown. C, The proportions of CD45\textsuperscript{+} CD11b\textsuperscript{+} CD11c\textsuperscript{+} DCs in the ear skin did not differ between Pdpn\textsuperscript{\textDelta LEC} and wild-type control mice. Mann–Whitney U test, P>0.05. Left, Pooled data from 3 independent experiments analyzing a total of 11 mice per genotype. Right, The mean value obtained in the 3 experiments. D, FACS analysis shows surface C-type lectin-like receptor 2 (CLEC-2) expression on LN DCs (gated on CD45\textsuperscript{+} MHCII\textsuperscript{+} CD11c\textsuperscript{+} cells), histograms, and delta median fluorescent intensities (MFI) are shown. Wild-type and Pdpn\textsuperscript{\textDelta LEC} mice express comparable levels of CLEC-2 (n=5).
hemostasis. Loss of CLEC-2 in the hematopoietic lineage using fetal liver chimeras, genetic approaches, or antibody treatment caused filling of the TD with blood from the subclavian vein; thus, these mice displayed blood-filled mesenteric LNs and vessels. Our study directly addresses the important role that podoplanin on LECs, as the molecular counterpart of CLEC-2, plays in controlling blood–lymphatic separation in developed animals.

Several reports described blood-filled LNs and Peyer patches in adult mice lacking CLEC-2 specifically in the megakaryocyte/platelet lineage or after treatment with CLEC-2 depleting antibodies. However, in models of CLEC-2 deficiency, LN bleeding was also described to occur because of compromised HEV integrity. It was suggested that podoplanin on FRCs interacts with CLEC-2–expressing platelets, inducing release of sphingosine-1-phosphate that tightens interendothelial junctions in HEVs. Our immunofluorescence analyses of frozen LN sections in PdpnΔLEC mice revealed blood cells inside LVs of the LNs, but never in close proximity to HEVs. Moreover, FRCs normally lined HEVs and retained podoplanin expression in PdpnΔLEC LNs. These observations indicate that the blood-filled LN phenotype was not caused by disruption of HEV integrity, but solely by blood filling into the LVs of the LNs.

The direct analysis of the lymphovenous valve and the presence of thrombi in its proximity has been recently studied by whole-embryo histology. However, the investigation of this anatomic structure by histology in adult animals is technically challenging because of its deep location in the upper chest, and to the best of our knowledge, has not been described to date. Our detailed anatomic analysis of the affected LNs and major LVs (ie, the TD) that constitute the terminal end of the lymphatic system, together with the published data of models of CLEC-2 deficiency, strongly supports our hypothesis that podoplanin functions at the interface between the lymphatic and the blood vascular system to prevent flow of blood into the lymphatic circulation postnatally (Figure V in the online-only Data Supplement).

Despite the presence of blood-filled LVs in the LN, the lymphatic drainage function was not compromised in PdpnΔLEC mice under steady-state conditions, as evaluated by the clearance of an injected tracer from ear skin. Moreover, we could perfuse a lymphatic tracer into the blood-filled TD of PdpnΔLEC mice after intradermal injections into the hind paws. Similarly, Hess et al observed fluorescence in the TD of CLEC-2–deficient animals after FITC-dextran injection in the hindlimb, suggesting that forward lymphatic flow was present even when blood filled the lymphatic system in a model of CLEC-2 deficiency. It is possible that this residual forward flow is sufficient to ensure physiological fluid drainage in PdpnΔLEC mice, and it would be of interest to test the drainage function in conditions characterized by increased demand of fluid drainage, such as inflammation. Another possible explanation could be that new alternate flow routes may be used to bypass the affected LNs, similar to what has been described in metastasis bearing LNs.

Surprisingly, PdpnΔLEC mice do not show signs of distress or lethality, despite the presence of blood in the LVs. It is not uncommon that the presence of profound lymphatic defects does not translate in lethality, a remarkable example is the K14-sVEGFR3 mouse line, which is viable despite completely lacking dermal LVs and displaying lymphedema. Importantly, we found a reduced DC migration to LNs in response to FITC challenge in PdpnΔLEC mice. It has been reported that CLEC-2 expressed by DCs interacts with podoplanin expressed by LECs and FRCs to induce actin cytoskeleton rearrangement and cell motility. Consequently, CLEC-2−/− fetal liver chimeras or mice lacking CLEC-2 specifically in CD11c+ DCs show reduced proportions of DCs migrating to the LNs. Here, we complement these observations made in CLEC-2–deficient mice and reinforce the importance of podoplanin expressed by LVs in DC migration. Importantly, we observed no differences in the number of migratory DCs present in PdpnΔLEC mice under steady-state conditions. Similarly, HEV maintenance, which has been linked to DC migration, was not affected. These data suggest that CLEC-2 affects DC migration in the context of induction of inflammation and they are in agreement with published data showing no constitutive CLEC-2 expression in resting DCs, but upregulation on lipopolysaccharide-induced systemic inflammation in a small subset of activated DCs. Taken together, our study highlights an important role of podoplanin in adult LVs; podoplanin prevents blood mixing into the lymphatic system postnatally and contributes to efficient DC migration to the LN in response to inflammation.

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Disclosures

None.

References


**Highlights**

- Generation of the first inducible, lymphatic-specific, podoplanin knockout mouse model (PdpnΔLEC mice).
- Postnatal loss of podoplanin in lymphatic endothelium results in blood-filled lymphatic vessels and lymph nodes without affecting lymphatic vessel patterning, morphology, and drainage function of lymphatic vessels or lymph node organization.
- PdpnΔLEC mice show impaired dendritic cell migration to lymph nodes.
- Podoplanin on adult lymphatic vessels plays important functions in maintaining lymph–blood separation and in contributing to efficient dendritic cell migration.
Postnatal Deletion of Podoplanin in Lymphatic Endothelium Results in Blood Filling of the Lymphatic System and Impairs Dendritic Cell Migration to Lymph Nodes

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Supplementary Figure I. Podoplanin is downregulated in LVs in different organs and at different time points in Pdpn\(^{\Delta LEC}\) mice. A-C. Confocal z-stack images of whole-mount preparations of abdominal skin (A), diaphragm (B) and mesentery (C) of P7 pups stained for LYVE-1 and podoplanin or, for the mesentery, podoplanin, CD31 and Prox1. Podoplanin was undetectable in LVs of P7 Pdpn\(^{\Delta LEC}\) mice in all organs analyzed. Scale bars: 100 μm. D. Confocal z-stack images of whole-mount preparations of ear skin samples from P21 animals stained for LYVE-1 and podoplanin revealed absence of podoplanin on LV in Pdpn\(^{\Delta LEC}\) mice. Scale bars: 100 μm. At least 5 animals per time point were analyzed.
Supplementary Figure II. Pdpn^{LEC} lymph nodes are blood-filled. A. Overview of the neck anatomy (left panels, scale bar: 1 mm) and image of the sub-mandibular lymph nodes (right panels, scale bars: 500 μm). B. Mesenteric lymph nodes, scale bar 1 mm. C Single color confocal images of frozen sections of axillary LNs stained for LYVE-1 (green), Ter119 (red) and CD31 (cyan), corresponding to the merged images shown in Figure 4F. Scale bars: 100 μm. D. Confocal images of frozen sections of a Pdpn^{LEC} LN stained for LYVE-1 (green), VE-cadherin (cyan) and Ter119 or a rat IgG control (red) showing the specificity of Ter119 for red blood cells. Scale bars: 100 μm.
Supplementary Figure III. Follicular reticular cells retain podoplanin expression. Confocal images of LN frozen sections stained for podoplanin (green), LYVE-1 (red) and CD31 (magenta). Arrowheads indicate podoplanin+ FRCs lining CD31+ HEVs, arrows indicate LVs. At least 6 mice per genotype were analyzed. Scale bars: 100 μm.
Supplementary Figure IV. Podoplanin is necessary to prevent blood-filling of the lymphatic system throughout life. **A.** Adult (>22 weeks old) Pdpn\textsuperscript{\textalpha LEC} mice (n=3) were administered tamoxifen intraperitoneally for 5 consecutive days and analyzed 5 days after the last injection. **B.** Confocal z-stack images of whole-mount ear skin samples show that podoplanin was downregulated on LYVE-1\textsuperscript{+} LVs in Pdpn\textsuperscript{\textalpha LEC} mice at the time of analysis; however, some vessels retained low podoplanin expression (arrows). Scale bars: 100 μm. **C.** In 1 out of 3 Pdpn\textsuperscript{\textalpha LEC} mice blood was detected in the thoracic duct (dashed line).
Supplementary Figure V. Hypothetical model of the retrograde flow of blood into the lymphatic vasculature. A. Schematic representation of the anatomy of a wild-type mouse. The lymphovenous valves are located at the connection of the thoracic duct and the subclavian veins and prevent blood from entering the lymphatic system. B. Pdpn^{ALEC} mice display blood-filled LNs and lymphatic vessels. Lack of podoplanin allows retrograde flow of blood into the lymphatic circulation. Blood reaches the LVs of the cervical and axillary regions first. Less frequently (dashed red vessels), blood is detected in the lower portion of the thoracic duct and at more distal sites, such as the mesenteric LNs and vessels, the lymphatic flank collector and inguinal LNs.
Supplementary Data to:

Bianchi et al.,

Postnatal deletion of podoplanin in lymphatic endothelium results in blood filling of the lymphatic system and impairs DC migration to lymph nodes

Materials and Methods:

Mouse lines and tamoxifen administration

Prox1-Cre-ERT2 mice\(^1\) (kindly provided by Dr. Taija Mäkinen) and Pdpn\(^{fl/fl}\) mice\(^2\) were postnatal intercrossed to obtain the Pdpn\(^{ALEC}\) line on the C57BL/6 background. To induce postnatal gene deletion, tamoxifen (Sigma) was dissolved in ethanol/sunflower seed oil (1:20 vol/vol) and administered intragastrically (50 µg per dose) to pups daily from postnatal day (P) 1 to P3. In another set of experiments, adult mice (at least 22 weeks of age) were administered tamoxifen intraperitoneally (1 mg per dose) for 5 consecutive days and analyzed 5 days after the last injection. Wild-type littermates (Pdpn\(^{wt/wt}\), Prox1-Cre-ERT2 or Pdpn\(^{fl/fl}\)) treated with the same regimen were used as controls. Both male and female mice were analyzed. Mice were sacrificed and analyzed at P7, P21 or when adults (10 to 23 weeks old). P7 animals were sacrificed by decapitation, adult animals were sacrificed by over-dose injection of a ketamine/medetomidine mixture (1000 mg/kg ketamine; 3.5 mg/kg medetomidine); no terminal bleeding or perfusion was performed. All mice used in this study were bred and housed in the SOPF animal facility of ETH Zurich and experiments were performed in accordance with animal protocols approved by the local veterinary authorities (Kantonales Veterinäramt Zürich).

Immunofluorescence and morphometric analyses of tissue whole-mounts

7-days-old pups were sacrificed and diaphragm, abdominal skin, tail skin and mesentery were collected. For skin analyses, samples were incubated for 2 hours at 37° C in 20 mM EDTA and the epidermal layer was removed. Ears were harvested from adult animals, hair was removed with depilation cream and ears were split into two halves along the cartilage. Samples were fixed for 2 hours in 4% paraformaldehyde at 4° C. After 1 hour wash in phosphate-buffered saline (PBS) and 4 hours incubation in blocking solution (5% normal donkey serum, 1% BSA, 0.01% Triton-X 100 in PBS), samples were incubated overnight with the following primary antibodies in blocking solution: hamster anti-podoplanin (1:50, clone 8.1.1, Developmental Studies Hybridoma Bank, University of Iowa), goat anti-podoplanin (1:200, R&D Systems), rabbit anti-LYVE-1 (1:600, Angiobio), rabbit anti-Prox1 (1:100, Angiobio), rat anti-CD31 (1:200, BD Pharmingen). After several hours washing in PBS, samples were incubated for 2 hours with appropriate secondary antibodies, conjugated with AlexaFluor-488, -594 or -647 fluorescent dyes (1:200, Invitrogen). After extensive washes in PBS, samples were flat-mounted on glass slides with Vectashield mounting media (Vector). Whole mount z-stacks were acquired using an LSM 710 FCS confocal microscope equipped with a 10x 0.3 NA EC Plan-Neofluar and a 20x 0.8 NA Plan-Apochromat objective using ZEN software (Zeiss), and maximum intensity projections were obtained with ImageJ software. Images of three randomly chosen individual fields of view from each LYVE-1 stained whole-mount skin sample were acquired. Two diaphragmatic segments per mouse were acquired. Computer-assisted morphometric analysis was performed using ImageJ software. Briefly, an area-calculating tool was used to quantify the total area covered by lymphatic vessels (LVs). The total LV length was quantified using a custom-made macro. The average vessel diameter was calculated by dividing the
total area covered by LVs by the total vessel length. The number of branch points and of ring structures was quantified manually.

**Immunofluorescence stainings of lymph node frozen sections**

LNs were embedded in OCT. Frozen sections (7 µm) were fixed in 4% paraformaldehyde, followed by permeabilization with 0.3% Triton X-100, or in -20°C acetone followed by rehydration in 80% methanol. Primary antibodies (goat anti-podoplanin, 1:100, R&D Systems; rabbit anti-LYVE-1, 1:600, Angiobio; rat anti-CD31, 1:62.5, clone MEC13.3, BD Pharmingen; hamster anti-CD31, 1:250, clone 2H8, Thermo Scientific; rat anti-Ter119, 1:150, eBioscience; goat anti-VE-cadherin, 1:100, R&D Systems; rat anti-B220, 1:200, clone RA3-6B2, BD Pharmingen; rat anti-TER119, 1:150, eBioscience; goat anti-VE-cadherin, 1:100, R&D Systems; rat anti-B220, 1:200, clone RA3-6B2, BD Pharmingen; rat anti-PNAd, 1:200, clone MECA79, BD Pharmingen) were applied in antibody diluent (Zytomed) or in PBS containing 5% normal donkey serum, 1% BSA and 0.01% Triton X-100 after 1 hour incubation in the same solution. After washes in PBS, AlexaFluor-488, -594 or -647 coupled secondary antibodies raised in donkey (1:200, Invitrogen) and nuclear counterstain (1:1000, Hoechst 33342, Invitrogen) were applied for 30 minutes. After washing, samples were mounted with Mowiol mounting medium.

Images were acquired using an LSM 780 confocal microscope equipped with a 10x 0.3NA EC Plan-Neofluar Ph1 M27 and a 20x 0.5NA EC Plan-Neofluar Ph2 M27 objective using ZEN software (Zeiss), and were processed with ImageJ software.

**Fluorescence-activated cell sorter (FACS) analysis**

Mouse ear skin was digested as previously described. LNs were mechanically disaggregated and red blood cells were lysed with ACK lysis buffer (BD Pharmingen). Tissue single cell suspensions were stained with the following antibodies after performing FcR blocking (rat anti-CD16/32, clone 93, Biolegend): rat anti-CD45-APC-Cy7 (clone 30F-11, Biolegend), rat anti-CD31-APC (clone MEC13.3, BD), hamster anti-podoplanin (clone 8.1.1) and anti-hamster-PE (Invitrogen), rat anti-LYVE-1-biotin (clone ALY7, eBioscience) and 488-coupled streptavidin (Invitrogen), rat anti-Ter119-FITC (Biolegend), rat anti-I-A/I-E-PerCp (clone M5/114.15.2, BD), hamster anti-CD11c-APC (clone N418, Biolegend), rat anti-CD11b-FITC (clone M1/70, Biolegend), rat anti-CLEC-2-PE (clone 17D9, Biolegend). FACS analyses were performed on a BD Canto analyzer (BD Biosciences) using the FACSDiva software. Data were analyzed with FlowJo software (TreeStar). LECs were gated as CD45+ CD31+ LYVE-1+ and the median fluorescence intensity for podoplanin was quantified and normalized to wild-type samples. Total LN cellularity and absolute numbers of cell populations were calculated with the aid of counting beads (Accu Check, Invitrogen).

**In vivo lymphatic clearance assay**

LV functionality was assessed by measuring the clearance over time of a pegylated near-infrared dye (P20D800) injected into the ear as previously described. Briefly, 3 µL of 3 µM P20D800 tracer were injected intradermally into the ears and imaging was performed using an IVIS spectrum (Caliper Life Sciences) at 0, 1, 2, 4, 6 and 24 hours after injection (λex: 745 nm, λem: 800 nm, binning of 2, exposure time 4 s). Fluorescence intensities from each ear were measured as fluorescence counts, baseline fluorescence was subtracted to calculate tissue enhancement values, and data were normalized to the intensity directly after injection. Data from each ear were fit to a one-phase exponential decay model in GraphPad Prism to generate the decay constant K (Normalized Tissue Enhancement = e^-kt, K expressed in hours^-1) and the half-life (Half Life = ln 2 / K, expressed in hours) as a measure of lymphatic clearance.

**Lymphatic imaging of the thoracic duct**
Mice were overdosed with anesthesia (1000 mg/kg ketamine; 3.5 mg/kg medetomidine) and the dorsal aspect of both footpads was intradermally injected with 10 µL of 20 µM P20D680 tracer. The wall of the thorax was surgically exposed to locate the thoracic duct. Anatomical (white light) and NIR images were acquired with a Zeiss StereoLumar.V12 (Zeiss) stereomicroscope equipped with a high-powered light emitting diode (LED) system with illumination at 635 nm (CoolLED), filters specific for Cy5 (Zeiss) and a CCD camera (Zeiss MRc). Images where overlaid using Photoshop.

**Wet and dry weight measurement of LNs**

Mandibular LNs from adult Pdpn\(^{ΔLEC}\) mice or wild-type littermates were harvested and their wet weight was measured. The dry weight was measured after incubating the LNs at 65°C for 24 hours. Fluid content was calculated with the following formula: \((\text{wet-dry})/\text{wet} \times 100 = \% \text{ fluid}\).

**FITC painting**

FITC painting experiments were performed as previously described. Briefly, ears of adult Pdpn\(^{ΔLEC}\) mice or wild-type littermates were painted with 15 µl FITC solution (5 mg/mL fluorescein isothiocyanate (Sigma) dissolved in dibutylphalate:acetone 1:1). After 18 hours, mice were sacrificed and the ear draining auricular LNs were harvested, mechanically disaggregated and analyzed by FACS in order to quantify the proportions of migratory FITC positive DCs (CD11c\(^+\)MHCII\(^+\)FITC\(^+\)) as % of total cellularity.

**Statistical analysis**

The unpaired Student’s t-test or the Mann-Whitney test were used to analyze normally or not normally distributed data sets, respectively. Results are shown as mean ± standard error of the mean (SEM). \(P<0.05\) was considered as statistically significant. Statistical analysis was performed with GraphPad Prism.

**References:**

Podoplanin is important for activated DC migration to the LN.

Podoplanin controls lymph-blood separation.

Pdpn<sup>ΔLEC</sup>: ↓ DC migration

Pdpn<sup>ΔLEC</sup>: blood filling of the lymphatic system

Legend:
- Pdpn
- CLEC-2

Activated DC

Lymphatic vessels

LYMPH NODE

Thoracic duct

Subclavian vein

Platelet

Lymphatic endothelial cell

Lympho-venous valve

Blood