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

Running title: Podoplanin in adult lymphatic vessels

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ABSTRACT

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 non-endothelial 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 ($Pdpn^{\Delta LEC}$) and induced gene deletion postnatally. $Pdpn^{\Delta LEC}$ mice were viable and their lymphatic vessels appeared morphologically normal with unaltered fluid drainage function. Intriguingly, $Pdpn^{\Delta LEC}$ 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 FACS analyses revealed normal lymph node organization with the presence of erythrocytes within lymph node lymphatic vessels but not surrounding high endothelial venules. Moreover, FITC painting experiments revealed reduced dendritic cell migration to lymph nodes in $Pdpn^{\Delta LEC}$ 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.

NON-STANDARD ABBREVIATIONS AND ACRONYMS

LN	lymph node
LV	lymphatic vessel
LVV	lymphovenous valve
TD	thoracic duct
LECs	lymphatic endothelial cells
FRCs	follicular reticular cells
CLEC-2	C-type lectin-like receptor 2
DCs	dendritic cells
HEVs	high endothelial venules

INTRODUCTION

The lymphatic vascular system is responsible for tissue fluid homeostasis, immune cell trafficking and intestinal lipid uptake.[1, 2] Peripheral lymphatic capillaries take up interstitial fluid (i.e. lymph) that is then filtered through lymph nodes (LN) and drained by lymphatic collecting vessels that converge into the thoracic duct (TD) - the largest lymphatic vessel (LV) in the body - and the right lymphatic trunk, which finally connect to the subclavian veins, where the lymph enters the blood circulation. At these connection points, specialized valves known as lymphovenous valves (LVV) prevent the flow of blood into the lymphatic circulation.[3, 4] LVs also play key functions in the trafficking of immune cells from the peripheral tissues to the draining LNs, where the initiation of adaptive immune responses occurs.[5]

Podoplanin is a small, extensively O-glycosylated, type-1 transmembrane protein expressed by lymphatic endothelial cells (LECs)[6] but not by blood vascular endothelial cells and is therefore a widely used lymphatic specific marker. Podoplanin is also expressed by follicular reticular cells (FRCs) of secondary lymphoid organs[7], podocytes of the kidney[8], alveolar type-1 cells of the lung and by the choroid plexus epithelium[9]. Moreover, it is expressed in the developing heart of the mouse embryo.[10] Podoplanin is the only described endogenous ligand of the C-type lectin-like receptor 2 (CLEC-2)[11], which is expressed by platelets[12] and activated dendritic cells (DCs)[13-15]. CLEC-2 signaling is involved in platelet activation[12], DC motility[15] and in the maintenance of vascular integrity[16], LN structure and development.[17]

Podoplanin global knockout mice die shortly after birth, due to a failure to inflate their lungs.[18, 19] Several studies described profound defects in the development and patterning of the lymphatic vasculature in these mice. Global podoplanin 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 which are replaced by blood-filled, poorly organized remnants.[24] Additionally, 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 thus far 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, for the first time, an inducible, lymphatic-specific podoplanin knockout mouse by crossing $Pdpn^{fl/fl}$ mice[28] and Prox1-Cre-ERT2 mice[29] to obtain $Pdpn^{\Delta LEC}$ mice. Postnatal deletion of podoplanin was compatible with life and did not affect the maintenance of normal LV morphology and function. Strikingly, however, blood was frequently detected in the thoracic duct (TD) of $Pdpn^{\Delta LEC}$ mice. Moreover, a subset of their LNs, despite maintaining a normal organization, were frequently blood-filled with detection of blood within the LVs, whereas no disruption of high endothelial venule (HEV) integrity was observed. Furthermore, DC migration to LNs after FITC challenge was reduced in $Pdpn^{\Delta LEC}$ mice, highlighting a role for podoplanin in immune cell trafficking to the LN. Taken together, these results suggest important postnatal functions of LV-expressed podoplanin in preventing

retrograde flow of blood into the lymphatic system and in regulating DC migration to LNs after challenge.

METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

Generation of a conditional, lymphatic-specific podoplanin knockout mouse model.
Since global constitutive podoplanin knockout mice display a lethal phenotype on most genetic backgrounds, at least in part due to a failure to inflate their lungs,[18] we generated mice with postnatal deletion of podoplanin specifically in the lymphatic vasculature. To this aim, $Pdpn^{fl/fl}$ mice[28] were crossed with Prox1-Cre-ERT2 mice[29], which express Cre recombinase under control of the lymphatic specification gene Prox1[30] upon tamoxifen administration, obtaining the $Pdpn^{\Delta LEC}$ 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 $Pdpn^{\Delta LEC}$ mice showed efficient podoplanin downregulation in dermal LVs, as assessed by whole mount immunofluorescence stainings for podoplanin and the lymphatic marker LYVE-1 (Figure 1B and SID), and by FACS analysis (Figure 1C). $Pdpn^{\Delta LEC}$ mice were monitored from birth until 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, $Pdpn^{\Delta LEC}$ 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 SIA-C). 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 $LYVE\text{-}1^{+}$ area, number of branch points, number of ring structures, LV diameter, total vessel length and average and branch length; Figure 2 B-D).

Adult lymphatic vessels 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-weeks-old mice were stained for LYVE-1. LV morphology appeared normal in adult $Pdpn^{\Delta LEC}$ 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[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 lymphatic clearance between $Pdpn^{\Delta LEC}$ mice and wild-type littermates (Figure 3B).

Postnatal deletion of podoplanin results in blood-filled lymphatic vessels and lymph nodes.
The LNs of adult $Pdpn^{\Delta LEC}$ mice often displayed a bloody appearance (Figure 4A, 4C and SIIA-B). In order 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 anatomical locations (Figure 4B), at P7 the LNs were never blood-filled and we only observed blood in the thoracic duct of 1 out 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 SIIB) and intestinal LVs (Figure 4D) were blood-filled only in adult Pdpn$^{\Delta LEC}$ mice and were not affected at P21 or P7.

FACS analysis revealed an increase in the frequency of red blood cells (identified as CD45^{-} Ter119^{+} events) in auricular LNs harvested from adult Pdpn$^{\Delta LEC}$ mice (7.7 ± 2.17% of cells), as compared to wild-type littermates (1.3 ± 0.53%), confirming the presence of blood in these LNs (Figure 4E).

To determine the origin of bleeding, we performed immunofluorescence analyses for red blood cells (Ter119), LYVE-1 and the pan-endothelial marker CD31 or VE-cadherin on frozen LN sections (Figure 4F and SIIC-D). Red blood cells were detected inside CD31^{+}/LYVE-1^{-} HEVs (Figure 4F, arrowheads), but also inside LVs in Pdpn$^{\Delta LEC}$ mice (Figure 4F, arrows and high power inset, Figure SIID). Importantly, no red blood cells were observed in the proximity of HEVs, suggesting that extravasation of erythrocytes from these vessels did not occur. Since FRCs lining HEVs retained podoplanin expression in Pdpn$^{\Delta LEC}$ mice (Figure SIII, arrowheads), the presence of blood in the LNs was not due to compromised HEV integrity, but to blood filling of the lymphatic vasculature.

We also analyzed the presence of blood in the TD of Pdpn$^{\Delta LEC}$ mice, which was identified by fluorescent lymphatic tracing after intradermal injection of the lymphatic tracer P20D680[31] in both hind paws. Blood was visible by white-light analysis in the TD in 36.4% (4 out of 11) of Pdpn$^{\Delta LEC}$ mice (Figure 4G) but not in wild-type mice (n=7). In the majority of Pdpn$^{\Delta LEC}$ mice (3 out 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 SIB). Taken together, these data suggest that lack of podoplanin on LECs results in retrograde flow of blood from the veins into the lymphatic system.

In order 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 SIVA). 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 SIVB). We did not observe any blood in the LNs, but in 1/3 mice blood filled the TD (Figure SIVC). 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 lymph node organization.
Global podoplanin knockout mice have poorly organized LNs.[24] To assess the contribution of LV podoplanin to LN organization, we analyzed the morphology of Pdpn$^{\Delta LEC}$ LNs in more detail. Histological analyses revealed normal B-cell follicle organization in Pdpn$^{\Delta LEC}$ LNs, as compared to wild-type controls (Figure 5A). Ear draining LNs of Pdpn$^{\Delta LEC}$ mice were heavier, while 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-F). We also analysed HEVs by immunostaining for the differentiation marker MECA-79 and observed a normal HEV morphology in LNs of Pdpn$^{\Delta LEC}$ mice (Figure 5G).
We found heavier LNs in Pdpn$^{\Delta LEC}$ 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 analysed 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 Pdpn$^{\Delta LEC}$ mice as compared to 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 Pdpn$^{\Delta LEC}$ mice.

Postnatal deletion of podoplanin impairs dendritic cell migration.
It has been suggested that CLEC-2 deficiency hampers DC migration to the LN.[15] To investigate if 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 Pdpn$^{\Delta LEC}$ mice as compared to 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 Pdpn$^{\Delta LEC}$ mice (Figure 6C). FACS analysis confirmed that LN DCs expressed CLEC-2 and that Pdpn$^{\Delta LEC}$ mice retained normal CLEC-2 expression (Figure 6D).

DISCUSSION

Podoplanin is a marker of lymphatic endothelium but is also expressed by other cell types such as LN FRCs and alveolar type-I cells in the lung (reviewed by Astarita et al.[33]). Thus, global constitutive podoplanin-deficient mice display a complex phenotype characterized by death at birth due to lung abnormalities, heart defects, lack of most LNs, edema and blood-filled LVs.[18, 20, 22, 24, 26] Here, for the first time, 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[11] and this biochemical interaction has been investigated in detail. The extracellular portion of podoplanin contains conserved domains required for CLEC-2 engagement[34], which in platelets results in the phosphorylation of the CLEC-2 cytoplasmic tail, activation of the Syk tyrosine kinase and consequent downstream signaling via SLP-76 and PLCγ2, leading to platelet activation.[11] 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 (reviewed by Bertozzi et al. [35]). Indeed, mice lacking either podoplanin, CLEC-2 or downstream signaling molecules such as Syk and SLP-76, all display a similar blood-filled lymphatic phenotype.[21-23, 36-39] 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.[40] We did not observe lethality or gross developmental defects up to 23 weeks of age in Pdpn$^{\Delta LEC}$ 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 Pdpn$^{\Delta LEC}$ 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.[20] Additionally, blocking of podoplanin function by administration of podoplanin-Fc during late embryonic development resulted in a less complex diaphragmatic lymphatic network in pups.[41] 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 lymphatic vessel 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$^{\Delta LEC}$ 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$^{\Delta LEC}$ mice was the presence of blood-filled LN LVs especially in the neck and axilla (95.5% and 68.2% of adult Pdpn$^{\Delta LEC}$ mice respectively) and, less frequently, flank collecting LVs (7.6% of adult Pdpn$^{\Delta LEC}$ mice) and mesenteric LNs and intestinal LVs (31.8% and 10.6% of adult Pdpn$^{\Delta LEC}$ mice respectively). Moreover, blood was present in the TD of 36.4% of adult Pdpn$^{\Delta LEC}$ mice analyzed. Interestingly, LN LVs were never blood-filled in P7 Pdpn$^{\Delta LEC}$ mice (i.e., 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, similarly to what we observed at P7 after tamoxifen injections at P1 to P3.

Moreover, the incomplete penetrance of this phenotype might be due to 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$^{\Delta LEC}$ mice. The observation that the LNs located in the neck and axilla, which are anatomically closer to the LVVs, 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 LVV, allowing filling of blood into the lymphatic system (Figure SV). Our observations confirm and extend a recent study that revealed an important function of platelet CLEC-2 in lymphovenous hemostasis.[45] 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.[45, 46] Our study, for the first time, 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's patches in adult mice lacking CLEC-2 specifically in the megakaryocyte/platelet lineage or after treatment with CLEC-2 depleting antibodies.[16, 17, 45] However, in models of CLEC-2 deficiency, LN bleeding was also described to occur due to compromised HEV integrity.[16] It was suggested that podoplanin on FRCs interacts with CLEC-2 expressing platelets, inducing release of sphingosine-1-phosphate that tightens inter-endothelial junctions in HEVs.[16] Our immunofluorescence analyses of frozen LN sections in Pdpn$^{\Delta LEC}$ 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 Pdpn$^{\Delta LEC}$ 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 LVV and the presence of thrombi in its proximity has been recently studied by whole-embryo histology [45]. However, the investigation of this anatomical structure by histology in adult animals is technically challenging due to its deep location in the upper chest, and to the best of our knowledge, has not been described so far. Our detailed anatomical analysis of the affected LNs and major lymphatic vessels (i.e. the TD) that constitute the terminal end of the lymphatic system, together with the published data of models of CLEC-2 deficiency [45], strongly support 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 SV).

Despite the presence of blood-filled LVs in the LN, the lymphatic drainage function was not compromised in $Pdpn^{\Delta LEC}$ 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 $Pdpn^{\Delta LEC}$ mice after intradermal injections into the hind paws. Similarly, Hess and colleagues observed fluorescence in the TD of CLEC-2 deficient animals after FITC-dextran injection in the hind limb, suggesting that forward lymphatic flow was present even when blood filled the lymphatic system in a model of CLEC-2 deficiency.[45] It is possible that this residual forward flow is sufficient to ensure physiological fluid drainage in $Pdpn^{\Delta LEC}$ 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, similarly to what has been described in metastasis bearing LNs.[31]

Surprisingly, $Pdpn^{\Delta LEC}$ 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 [47].

Importantly, we found a reduced DC migration to LNs in response to FITC challenge in $Pdpn^{\Delta LEC}$ 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.[15] Consequently, $CLEC\text{-}2^{-/-}$ fetal liver chimeras or mice lacking CLEC-2 specifically in $CD11c^{+}$ DCs show reduced proportions of DCs migrating to the LNs.[15, 48] 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 $Pdpn^{\Delta LEC}$ mice under steady-state conditions. Similarly, HEV maintenance, which has been linked to DC migration[49], was not affected. These data suggest that CLEC-2 impacts on DC migration upon induction of inflammation and they are in agreement with published data showing no constitutive CLEC-2 expression in resting DCs, but upregulation upon LPS-induced systemic inflammation in a small subset of activated DCs.[14]

Taken together, our study highlights an important role of podoplanin in adult lymphatic vessels: podoplanin prevents blood mixing into the lymphatic system postnatally and contributes to efficient DC migration to the lymph node in response to inflammation.

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DISCLOSURES

None.

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HIGHLIGHTS

- Generation of the first inducible, lymphatic-specific, podoplanin knockout mouse model ($Pdpn^{\Delta LEC}$ 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
- $Pdpn^{\Delta LEC}$ 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.

FIGURE LEGENDS

Figure 1. Generation of conditional, lymphatic-specific podoplanin knockout mice. A. $Pdpn^{fl/fl}$ mice were crossed with Prox1-Cre-ERT2 mice to obtain the $Pdpn^{\Delta LEC}$ line. Postnatal gene deletion was induced by tamoxifen administration from postnatal day (P)1 to P3. **B.** Confocal z-stack images of whole-mount ear skin samples show that podoplanin was undetectable on LYVE-1^+ LVs in adult $Pdpn^{\Delta LEC}$ animals. Scale bars: 100 µm. **C.** Ear skin single cell suspensions were analyzed by FACS. LECs were gated as $CD45^-$ $CD31^+$ LYVE-1^+ events. Representative histograms of podoplanin expression on LECs isolated from adult $Pdpn^{\Delta LEC}$ and wild-type control mice are shown. The median fluorescence intensity (MFI) for podoplanin in the LEC gate was significantly lower in $Pdpn^{\Delta LEC}$ samples. Mann-Whitney test, $P<0.001$. At least 6 mice per genotype were analyzed.

Figure 2. Postnatal deletion of podoplanin does not interfere with lymphatic patterning. A. Comparable body weights of $Pdpn^{\Delta LEC}$ mice and wild-type littermates at P7. Unpaired Student's t-test, P>0.05. **B.** Confocal z-stack images of representative whole-mounts of abdominal skin samples stained for LYVE-1 (green). Quantification of the LYVE-1^+ area and number of branch points per image showed no difference between genotypes. Mann-Whitney test, $P>0.05$. **C.** Confocal z-stack images of representative tail skin whole-mount samples stained for LYVE-1. Quantification of the LYVE-1^+ area and the number of ring-like structures per image revealed no difference between genotypes. Mann-Whitney test, $P>0.05$. **D.** Confocal z-stack images of representative diaphragm segments stained for LYVE-1. Quantification of the LYVE-1^+ 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 test, *P*>0.05. Four to five animals per genotype from 4 independent litters were analyzed. Scale bars: 100 μm.

Figure 3. Adult lymphatic vessels are morphologically and functionally normal after postnatal deletion of podoplanin. A. Confocal z-stack images of ear skin samples whole-mounts stained for LYVE-1 (red). Scale bar: 100 μm. Quantification of the LYVE-1$^+$ area, number of branches, total vessel length and average vessel diameter showed no difference between genotypes. Mann-Whitney test, *P*>0.05. Six animals per genotype from independent litters were analyzed. **B.** IVIS images of ears injected intradermally with P20D800 tracer at several time points after injection. Clearance of the tracer by LVs was calculated as half-life and K rate and showed no difference between the two groups. Unpaired Student's t-test, *P*>0.05. Each data point represents one injected ear. Six to eight animals per genotype were analyzed.

Figure 4. Postnatal deletion of podoplanin results in blood-filled LVs and lymph nodes. A. Photograph of auricular LNs harvested from adult mice. Pdpn$^{\Delta LEC}$ LNs appear blood-filled. Scale bar: 1 mm. **B.** Frequency of blood-filled LNs of the neck (auricular and mandibular), axilla (axillary and brachial), mesentery (mes) and inguinal (ing) regions and thoracic duct (TD) in Pdpn$^{\Delta LEC}$ mice at different time points (P7 n=9; P21 n=5; adults n=66, n=11 for TD analysis; nd: not determined). **C.** Normal anatomy of the lymphatic flank collector and representative photograph of a Pdpn$^{\Delta LEC}$ mouse displaying filling of the collector (arrowheads) with blood, from the axillary (ax) LN to the inguinal (ing) LN. Scale bars: 5 mm. **D.** Normal anatomy of the adult mesentery and representative photograph of a Pdpn$^{\Delta LEC}$ mouse with blood-filled intestinal LVs. Scale bars: 1 mm (inset: 100 μm). **E.** Representative FACS plots of single cell LN suspensions stained for the red blood cell marker Ter119 and the leukocyte marker CD45. The frequency of red blood cells in Pdpn$^{\Delta LEC}$ LNs was significantly higher than in wild-type LNs. Mann-Whitney test, ***P*<0.01 **F.** Confocal images of frozen sections of axillary LN stained for LYVE-1 (green), Ter119 (red) and CD31 (cyan). Red blood cells (red) filled LN LVs (green; arrows) in Pdpn$^{\Delta LEC}$ samples. Red blood cells were also found inside HEVs (cyan; arrowheads) but never in their proximity. A minimum of 6 animals per genotype and condition were analyzed. Boxed area corresponds to higher power inset. Scale bars: 100 μm (inset: 25 μm). **G.** The fluorescent tracer P20D680 was used to identify the thoracic duct after injection into both hind paws. Blood was present in the TD of 36.4 % of Pdpn$^{\Delta LEC}$ mice, scale bar: 1 mm.

Figure 5. Postnatal deletion of podoplanin does not compromise lymph node organization. A. Confocal tile-scan of axillary LNs stained for the B cell marker B220 and with DAPI shows normal B-cell follicle organization in Pdpn$^{\Delta LEC}$ mice. Scale bars: 200 μm. **B.** Auricular LNs were significantly heavier in Pdpn$^{\Delta LEC}$ mice. Unpaired Student's t-test, ***P*<0.01. **C.** Total cellularity after red blood cell lysis showed no difference between genotypes. Mann-Whitney test, *P*>0.05. **D.** Total number of leukocytes (CD45$^+$ cells) per LN was not different between genotypes. Mann-Whitney test, *P*>0.05. **E.** Proportions of T cells (% of CD3$^+$ cells in CD45$^+$ gate) did not differ between genotypes. Mann-Whitney test, *P*>0.05. **F.** Total number of migratory DCs (CD45$^+$ MHCII^{high} CD11c$^+$ cells) did not differ between genotypes. Mann-Whitney test, *P*>0.05. **G.** Representative confocal images of LN frozen sections stained for MECA79 (red) and podoplanin (green) revealed normal HEVs in Pdpn$^{\Delta LEC}$ mice. Scale bars: 10 μm. **H.** Wet and dry weight of mandibular LNs were measured to calculate the fluid content. There was no difference in LN fluid content between the genotypes. Unpaired Student's t-test, *P*>0.05.

Figure 6. Postnatal deletion of podoplanin impairs dendritic cell migration to LNs. A. Schematic representation of the FITC painting experiment: FITC solution was applied to the ears, and the draining auricular LNs were harvested and analyzed by FACS after 18 hours. **B.** Representative FACS dot plots (gated on $MHCII^+$ events) showing $MHCII^+$ $CD11c^+$ $FITC^+$ DCs in wild-type and $Pdpn^{\Delta LEC}$ mice 18 hours after FITC application. Quantification of the proportions of $MHCII^+$ $CD11c^+$ $FITC^+$ DCs in the auricular draining LNs 18 hours after FITC application showed a significant reduction in $Pdpn^{\Delta LEC}$ mice. Mann-Whitney test, $*P<0.05$. Pooled data from 2 independent experiments analyzing a total of 7 mice per genotype are shown. **C.** The proportions of $CD45^+$ $CD11b^+$ $CD11c^+$ DCs in the ear skin did not differ between $Pdpn^{\Delta LEC}$ and wild-type control mice. Mann-Whitney test, $P>0.05$. Left panel shows pooled data from 3 independent experiments analyzing a total of 11 mice per genotype. Right panel shows the mean value obtained in the 3 experiments. **D.** FACS analysis shows surface CLEC-2 expression on LN DCs (gated on $CD45^+$ $MHCII^+$ $CD11c^+$ cells), histograms and delta median fluorescent intesities are shown. Wild-type and $Pdpn^{\Delta LEC}$ mice express comparable levels of CLEC-2 (n=5).