**LETTER**

Podoplanin maintains high endothelial venule integrity by interacting with platelet CLEC-2

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Circulating lymphocytes continuously enter lymph nodes for immune surveillance through specialized blood vessels named high endothelial venules1–4, a process that increases markedly during immune responses. How high endothelial venules (HEVs) permit lymphocyte transmigration while maintaining vascular integrity is unknown. Here we report a role for the transmembrane O-glycoprotein podoplanin (PDNP, also known as gp38 and T1α)5–7 in maintaining HEV barrier function. Mice with postnatal deletion of Pdpat lost HEV integrity and exhibited spontaneous bleeding in mucosal lymph nodes, and bleeding in the draining peripheral lymph nodes after immunization. Blocking lymphocyte homing rescued bleeding, indicating that PDNP is required to protect the barrier function of HEVs during lymphocyte trafficking. Further analyses demonstrated that PDNP expressed on fibroblastic reticular cells7,8, which surround HEVs, functions as an activating ligand for platelet C-type lectin-like receptor 2 (CLEC-2, also known as CLEC1B)9,10. Mice lacking fibroblastic reticular cell PDNP or platelet CLEC-2 exhibited significantly reduced levels of VE-cadherin (also known as CDH5), which is essential for overall vascular integrity11,12, on HEVs. Infusion of wild-type platelets restored HEV integrity in Clec-2-deficient mice. Activation of CLEC-2 induced release of sphingosine-1-phosphate13,14 from platelets, which promoted expression of VE-cadherin on HEVs ex vivo. Furthermore, draining peripheral lymph nodes of immunized mice lacking sphingosine-1-phosphate had impaired HEV integrity similar to Pdpat- and Clec-2-deficient mice. These data demonstrate that local sphingosine-1-phosphate release after PDNP–CLEC-2-mediated platelet activation is critical for HEV integrity during immune responses.

Lymph nodes (LNs) are essential sites for immune responses. They are organized into lobules, which are surrounded by lymphatic sinuses that deliver antigens fromafferent lymphatic vessels to LNs for identification by naive lymphocytes that continually home through HEVs (Supplementary Fig. 1). Lymphocyte trafficking is particularly prominent in mucosal LNs, as most foreign antigens enter the body through mucosal epithelium, and in draining peripheral LNs during immune responses15,16. How HEVs accommodate a high rate of lymphocyte trafficking while maintaining their integrity remains unknown.

Platelets support vascular integrity in inflamed tissues by still undefined mechanisms17. Whether, and if so how, platelets protect HEV integrity in the LN is unexplored. PDNP, a ligand for the platelet activating receptor CLEC-2, is highly expressed in LNs. We developed mice with tamoxifen-inducible global deletion of PDNP (Pdpat/−;CagCre, Supplementary Fig. 2a–d), and focused first on the mucosal LNs of mice around the weaning-age because development of adaptive immunity occurs early and primarily through mucosal LNs18. Tamoxifen administration from postnatal day 1 (P1) to P5 resulted in ~90% reduction of PDNP at P15 and complete loss at 1 month in both mucosal and peripheral LNs (Supplementary Figs 2e and 3a, b, d). Beginning at P15 and progressively worsening, Pdpat/−;CagCre pups exhibited massive bleeding primarily in mucosal LNs including mesenteric LNs (MLNs) and cervical LNs (CLNs), but rarely in peripheral (inguinal and popliteal) LNs (Fig. 1a, Supplementary Fig. 3). Histology and confocal imaging of MLNs revealed large numbers of extravasated red blood cells (RBCs) around HEVs but not non-HEV vessels of Pdpat/−;CagCre mice (Fig. 1b–d). Pdpat deletion starting at 3–4 weeks of age resulted in a similar mucosal LN bleeding phenotype, suggesting that PDNP is also important for LN vascular integrity in adults (Supplementary Fig. 3c, d).

In LNs, PDNP is expressed by endothelial cells of lymphatic vessels but not by blood vessels including HEVs (Supplementary Fig. 4a). However, PDNP is also highly expressed on fibroblastic reticular cells (FRCs), which surround HEVs and express ER-TR7, αSMA and PDGFRβ (Supplementary Fig. 4b–d). To address whether PDNP on FRCs is essential for LN vascular integrity, we generated Pdpat/−;PdgfrbCre mice, which lack PDNP in FRCs but otherwise exhibit normal RORC function (Supplementary Fig. 5b, e). Similar to Pdpat/−;CagCre mice, Pdpat/−;PdgfrbCre mice developed bleeding in mucosal LNs (Fig. 1e). Pdpat−/−;PdgfrbCre mice also had reduced levels of PDNP on lymphatic endothelial cells (LEC) in LNs (Supplementary Fig. 5b–d). To rule out the contribution of endothelial PDNP to LN bleeding, we developed Pdpat−/−;Tie2Cre mice, which lack PDNP specifically in LECs but not in FRCs (Supplementary Fig. 6a–d). Consistent with the previously described role of PDNP in the separation of blood and lymphatic vessels during embryonic development, Pdpat−/−;Tie2Cre mice exhibited blood–lymphatic vessel mixing phenotype (Supplementary Fig. 6c, data not shown). However, Pdpat−/−;Tie2Cre mice did not exhibit bleeding around HEVs in the LN (Supplementary Fig. 6e, f). These results indicate that PDNP on FRCs rather than LECs prevents bleeding in LNs.

CLEC-2 is the only known receptor for PDNP9,10,19. To determine its importance for LN vascular integrity, we depleted CLEC-2 in wild-type neonates using a CLEC-2-specific monoclonal antibody, INU1 (ref. 19). Administration of INU1 resulted in bleeding in mucosal LNs at P15 similar to Pdpat−/−;CagCre mice (Fig. 1f, Supplementary Fig. 7a). To determine the role of CLEC-2 in adult LNs, we made Clec-2−/− bone marrow chimeraes (CLEC-2−/−/BM chimerae, Supplementary Fig. 7b, c), which, consistent with deleting Pdpat in adult mice, developed bleeding in mucosal LNs (Supplementary Fig. 7d). CLEC-2 is primarily expressed on platelets14,19; however, it is also expressed on myeloid and dendritic cells. To verify whether CLEC-2 on platelets is required to protect LN vascular integrity, we developed mice lacking CLEC-2.

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specifically on platelets (Clec-2f/f;Pf4Cre, Supplementary Fig. 8a–c). Clec-2f/f;Pf4Cre mice developed spontaneous bleeding in mucosal LNs reminiscent of Pdpn-deficient mice (Fig. 1g, Supplementary Fig. 8d). Collectively, these results indicate that FRC PDPN and platelet CLEC-2 are required to protect LN vascular integrity.

To explore where platelet CLEC-2 interacts with PDPN in the LN, we stained cryosections of LNs and found frequent associations of platelets with PDPN+ FRCs at the abluminal sides of HEVs of wild-type but not Pdpn-deficient mice (Fig. 2a, Supplementary Fig. 9). Furthermore, intravenously transfused, fluorescently labelled CLEC-2-expressing wild-type platelets were detected on the abluminal sides of HEVs in close association with PDPN+ FRCs of Clec-2f/f;BM chimaeras (Fig. 2b), supporting the hypothesis that platelets interact with FRCs around HEVs in a PDPN–CLEC-2-dependent manner.

Mucosal LNs but not peripheral LNs of Pdpn- or Clec-2-deficient mice exhibit spontaneous bleeding, suggesting phenotypic differences between these two types of LNs. We found that mucosal LNs were significantly more permeable to Evans blue dye than peripheral LNs in the steady-state (Fig. 2c). We noted that mucosal but not peripheral HEVs express MADCAM-1 (Supplementary Fig. 10a, b). However, after an immune challenge (ovalbumin/complete Freund’s adjuvant, HEVs express MAdCAM-1 (Supplementary Fig. 10a, b). However, these data support the model that interactions between platelets and FRCs are critical for preventing bleeding in ‘reactive’ LNs during immune responses when lymphocyte trafficking is increased.

To determine whether impaired HEV barrier function is responsible for the observed LN bleeding when PDPN is absent, we intravenously injected fluorescein isothiocyanate (FITC)-conjugated dextran immediately before euthanasia. FITC–dextran was contained within HEVs of wild-type MLNs. However, FITC–dextran leaked from HEVs, but not from non-HEV blood vessels in LNs and other organs, of Pdpn;CagCre mice (Fig. 3a, Supplementary Fig. 12a and data not shown). Furthermore, intravenously injected, fluorescently labelled RBCs were detected outside of HEVs in Pdpn;CagCre but not in wild-type mice (Fig. 3b). Ultrastructural analyses indicated that abnormal gaps between endothelial cell membranes were specific to HEVs of Pdpn;CagCre mice, and RBCs were frequently observed between or outside of high endothelial cells in Pdpn;CagCre mice (Fig. 3c, Supplementary Fig. 12b). Together, these data indicate that, in the absence of PDPN, impaired vascular barrier function and defective junctions are restricted to HEVs.

VE-cadherin (VE-cad) is an integral component of endothelial adherens junctions and barrier function. We found that levels of VE-cad were reduced in HEVs of Pdpn;CagCre LNs starting at P8, before the onset of bleeding (Fig. 3c, Supplementary Fig. 12c, d), consistent with the idea that loss of HEV junctional integrity contributes to the onset of bleeding. Furthermore, following the loss of PDPN, β-catenin, another component of endothelial adherens junctions, was decreased...
Figure 2 | FRC PDNP and platelet CLEC-2 protect LN vascular integrity during immune responses. a, Confocal images of MLN cryosections stained with antibodies to PNAd, platelets (plts) and PDNP. Arrows indicate platelets on the abluminal side of HEVs. Arrowheads indicate platelets that are not associated with HEVs. Bar graph on the right represents percentage of HEVs with platelets on their abluminal side (mean ± s.d., 250 HEVs per mouse, n = 3). b, Confocal images of Pdpnf–/– HEVs, PDNP, and transfused fluorescently labelled wild-type platelets in MLNs. Arrows indicate platelets on the abluminal side of HEVs. c, Comparison of Evans blue permeability between MLNs and popliteal LNs (PLNs) of 1-month-old wild-type mice, 5 min after intravenous injections (mean ± s.d., n = 15 per group). d, Gross morphology (insets) and confocal images of PLNs after OVA/CFA challenge. e, Gross morphology (insets) and confocal images of CLNs from 3-week-old mice with (Pdpnf–/–;PdgfbCre) or without (Pdpnf–/–;PdgfbCre/Rag1–/–) lymphocytes. f, Gross morphology (insets) and confocal images of PLNs from 1-month-old mice, 1 week after OVA/CFA challenge and injections with a monoclonal antibody (Mel-14) that blocks L-selectin-dependent lymphocyte homing or with an isotype control rat IgG. Data represent at least 8 LNs per group from at least three experiments. Scale bars, 2 mm (light microscopy), 50 µm (confocal). Asterisk indicates bleeding and arrows mark extravasated RBCs around HEVs (d–f). ***p < 0.001.

Figure 3 | Interactions between FRC PDNP and platelet CLEC-2 are critical for HEV junctional integrity. a, Confocal images of intravenously injected FITC–dextran (2,000 kDa) in P15 MLNs. Arrows indicate vascular leak of FITC–dextran. Insets show non-HEV blood vessels. b, Confocal images of intravenously injected RBCs (fluorescently labelled, red) in MLNs from 2-month-old mice. Arrow shows labelled RBCs outside of HEVs (CD31+). c, Transmission electron micrographs of HEVs in MLNs. Arrow indicates gaps and RBCs between high endothelial cells. d, Confocal images of VE-cad in P8 MLNs. Insets show VE-cad staining in non-HEV blood vessels. e, Confocal analysis of intravenously injected FITC–dextran in MLNs from wild-type or Clec2–/– BM chimaeras with or without previous transfusions of wild-type platelets. Arrows indicate vascular leak of FITC–dextran. Inset depicts non-HEV blood vessels. Graphs on the right quantify number of leaking vessels (mean ± s.d., 300 vessels per group, n = 3). f, Anti-VE-cad staining of HEVs and non-HEV blood vessels in wild-type BM chimaeras, and Clec2–/– BM chimaeras without or with previous transfusions with wild-type platelets. Dagger indicates nonspecific staining as also observed in isotype controls. Graphs on the right quantify VE-cad staining (mean ± s.d., 300 vessels per group, n = 3). Tissues were from 1-month-old (a–c) or 12-week post-BM transplantation (e, f) mice unless otherwise specified. Dashed lines mark HEVs. Data represent at least three individual experiments. Scale bars, 2 µm (transmission electron microscopy), 50 µm (confocal images (inset a, b and d, 25 µm)). ***p < 0.001.
(Supplementary Fig. 12e) and expression of N-cadherin, which is essential for endothelial–stromal cell junctions\(^1\), was reduced around HEVs (Supplementary Fig, 12f). Further analyses revealed that VE-cad was expressed at higher levels in peripheral HEVs than in mucosal HEVs of wild-type mice (Supplementary Fig. 13a). ZO-1 (also known as TJP1)\(^2\), another junction molecule, was detected on peripheral but not mucosal HEVs. However, HEVs in draining peripheral LNcs of immunized wild-type mice exhibited reduced VE-cad and ZO-1 expression, resembling MLNs of wild-type mice (Supplementary Fig. 13a, b). Consistent with the bleeding in draining peripheral LNcs of immunized Pdpn\(^{-}\) or Clec-2\(^{-}\)-deficient mice (Fig. 2d), these LNcs exhibited a further reduction of VE-cad on their HEVs (Supplementary Fig. 13a), which was normalized by blocking lymphocyte trafficking with Mel-14 (Supplementary Fig. 13c). Furthermore, blocking VE-cad increased permeability in draining peripheral LNcs of immunized wild-type mice (Supplementary Fig. 14). These results support the idea that interactions of PDPN and CLEC-2 preserve HEV barrier function primarily by promoting VE-cad expression on HEVs.

HEVs of Clec-2\(^{-}\)/BM chimaeras exhibited increased permeability to intravenously injected FITC–dextran (Fig. 3e). To test whether this phenotype could be rescued by wild-type platelets, Clec-2\(^{-}\)/BM chimaeras were transplanted daily with wild-type platelets for 4 days before euthanasia. HEVs of Clec-2\(^{-}\)/BM chimaeras that received wild-type platelets exhibited significantly decreased permeability to FITC–dextran compared to controls (Fig. 3e). Notably, wild-type and Clec-2\(^{-}\)/BM chimaeras transplanted with wild-type platelets expressed comparable levels of VE-cad on HEVs (Fig. 3f, Supplementary Fig. 15). Together, these results demonstrate that PDPN and platelet CLEC-2 are essential for preserving adherens junctions on HEVs.

Platelet aggregation is essential for haemostasis\(^3\),\(^4\). However, blocking aggregation with a monoclonal antibody to integrin \(\alpha\)IIb\(\beta\)3 did not increase permeability in draining peripheral LNcs of immunized wild-type mice (Supplementary Fig. 14). This suggests that a platelet function mediated by PDPN–CLEC-2 apart from aggregation is required for HEV integrity. Sphingosine-1-phosphate (S1P)\(^1\),\(^2\),\(^3\),\(^4\), a bioactive lipid, is a strong candidate for mediating this platelet function as it is known to regulate vascular integrity through interactions with its G-protein-coupled receptors on endothelial cells\(^3\).

Although platelets generate and store S1P\(^5\), whether PDPN–CLEC-2-mediated platelet activation causes S1P release is unknown. To test this, we stimulated wild-type and Clec-2\(^{-}\/-\) platelets with the monoclonal antibody INU1, which activates CLEC-2 signalling \(\text{in vitro}\), and observed a CLEC-2-dependent S1P release from platelets (Fig. 4a). Furthermore, PDPN\(^+\) but not PDPN\(^-\) melanoma cells induced the release of S1P from wild-type but not Clec-2\(^{-}\/-\) platelets (Supplementary Fig. 16a, b), indicating that interactions between PDPN and CLEC-2 induce S1P release from platelets. Next, we found that MLN slices cultured \(\text{ex vivo}\) with fetal bovine serum (FBS), but not lipid-depleted FBS, retained VE-cad expression on their HEVs (Fig. 4b, Supplementary Fig. 16c), supporting the idea that lipids, such as S1P, maintain HEV adherens junctions. The addition of wild-type but not Clec-2\(^{-}\/-\) platelets rescued VE-cad expression on HEVs of MLN slices cultured in lipid-depleted FBS-containing media. Wild-type platelets were unable to restore HEV VE-cad expression on MLN slices from Pdpn\(^{-}\)deficient mice (Fig. 4b, Supplementary Fig. 16c), demonstrating that FRC PDPN and platelet CLEC-2 are required for normal VE-cad expression on HEVs. The increase of VE-cad on HEVs in the presence of wild-type platelets is due, at least in part, to the activation of the S1P

**Figure 4 | S1P release from platelets after PDPN–CLEC-2-dependent activation contributes to HEV barrier function.** a, S1P concentrations in supernatants of wild-type and Clec-2\(^{-}\/-\) platelets after incubation with CLEC-2 activating antibody, INU1 or isotype control (sham-treated). S1P in platelet lysates was used as the positive control (mean ± s.d., \(n = 4\) mice per group representing two individual experiments). b, Representative images of VE-cad staining of HEVs from wild-type LN slices incubated for 1.5 h with DMEM and normal FBS (media), DMEM and lipid-depleted FBS, lipid-depleted and wild-type platelets, lipid-depleted and Clec-2\(^{-}\/-\) platelets, lipid-depleted and S1P\(^-\) platelets, or lipid-depleted and wild-type platelets plus S1P antagonist W146. Pdpn\(^{-}\)/CagCre LN slices incubated with lipid-depleted and wild-type platelets were controls. One hundred HEVs were analysed per condition. Dashed lines mark HEVs. Daggers marks nonspecific staining as also observed in isotype controls. Graphs represent ratios of VE-cad intensities on HEVs relative to that of wild-type lymph node slices cultured with media (mean ± s.d., \(n = 20\) HEVs per group). c, Gross morphology (insets) and confocal images of draining PLNs after immunization. Asterisk indicates bleeding. Arrow marks bleeding (Ter119\(^+\)) around an HEV (CD31\(^+\)). d, Confocal images of PLN HEVs from S1P\(^-\) mice transfused with wild-type or Clec-2\(^{-}\/-\) platelets for 4 days after intravenous FITC–dextran injection. Arrows indicate vascular leak of FITC–dextran. Graphs on the right quantify leaking HEVs (mean ± s.d., \(50\) HEVs per group, \(n = 3\)). e, Model depicting how PDPN maintains HEV integrity during lymphocyte trafficking. FRC PDPN engages CLEC-2 on extravasated platelets in the perivascular space of HEVs and induces local release of S1P, which promotes VE-cad expression on the wild-type HEV (left). In contrast, loss of the interaction results in impaired HEV integrity and subsequent bleeding (right).

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lymphocytes (Supplementary Fig. 17a). However, after OVA/CFA transfusion for four consecutive days of wild-type but not Clec-2−/− mice exhibited bleeding, increased permeability to FITC–dextran, and vascular leak of injected FITC–dextran from HEVs of draining peripheral LNs of S1P−/− mice (Fig. 4c, d, Supplementary Fig. 17b–d). A daily transfusion for four consecutive days of wild-type but not Clec-2−/− platelets resulted in higher levels of VE-cadherin and reduced vascular leak of injected FITC–dextran from HEVs of draining peripheral LNs of immunized S1P−/− mice (Fig. 4d, Supplementary Fig. 18). Taken together, these data indicate that PDPN–CLEC-2–dependent local release of S1P from platelets plays a critical role in maintaining HEV integrity during immune responses.

Unlike other venules, HEVs are not circumscribed by typical pericytes and collagen-containing matrix that would activate platelets. Instead, they are surrounded by a perivascular sleeve of FRCs that sequester collagen fibres. Our findings reveal an important new role for FRC PDPN, which is well positioned to interact with CLEC-2 on extravasated platelets (Fig. 4e). S1P in the blood is known to regulate vascular permeability in situations of increased lymphocyte trafficking such as chronic inflammation. Recently, components of the CLEC-2 signalling pathway have been implicated in preventing inflammation-induced haemorrhage in the skin and lung. Therefore, PDPN–CLEC-2–mediated local S1P release from platelets may protect vascular integrity in other inflamed tissues.

**METHODS SUMMARY**

Mice were maintained in specific-pathogen-free facilities and used under protocols approved by the IACUC of the Oklahoma Medical Research Foundation. For all experiments, a minimum of six mutants of each line and littermates were examined unless otherwise stated based on the highly penetrant phenotypes observed in our preliminary studies. Data are expressed as mean ± s.d. and represent at least three experiments unless otherwise specified. Statistical analysis was performed with Student’s t-tests and differences were considered significant when P < 0.05. Generation of novel mouse lines, bone marrow transplantation, depletions of CLEC-2, platelet transfusion, immune challenge, L-selectin blocking, permeability assays, platelet and RBC labelling/transfusions, immunofluorescence imaging, flow cytometry, electron microscopy, S1P concentration analysis and ex vivo LNC experiments are described in detail in the Methods.

**Full Methods** and any associated references are available in the online version of the paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** B.H.H. and J.F. designed and performed experiments, analysed results and wrote the manuscript. J.M.M., Y.P., M.S., A.M. and F.L. performed experiments. S.J.W., R.H.A.S., F.M. and S.N. supplied key reagents and mice. R.P.M., H.C. and M.L.K. helped analyse results and commented on the manuscript. L.X. designed and supervised research and wrote the manuscript.

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METHODS

Mice. To generate mice with loxP-flanked Pdpn alleles (Pdpnfl/2), a targeting vector was constructed in which exon 2, the major coding exon of Pdpn gene, was flanked by loxP sites (Pdpnfl/2), and the neomycin resistance selection cassette (Neo) was flanked by Frt sites (Supplementary Fig. 2a). The NotI-linearized construct was electroporated into C57BL/6-derived embryonic stem (ES) cells, and correctly targeted clones were identified by Southern blots. The Frt-flanked Neo was removed by transient expression of Flp recombinase in the positive ES clones to avoid potential undesirable effects of the Neo cassette. ES cells with normal karyotype bearing a floxed Pdpn allele were microinjected into B6/Tyr blastocysts, which were subsequently implanted into pseudopregnant foster mothers. Male chimaeras were bred with C57BL/6J females for germline transmission. Heterozygous mice bearing a floxed Pdpn allele are flanked by loxP (Tg[Pdpnfl/2]) respectively.

To generate mice with inducible deletion of PDPN (Pdpnfl/2,CagCre), Pdpnfl/2 mice were crossed with the CAG-Cre-ERT2 Tg mice (B6.Cg-Tg(CAG-cre/Esrt1)5Amc/J, Jackson Laboratories)29. To induce postnatal deletion of PDPN, tamoxifen (MP Biomedical) was dissolved in ethanol/sunflower oil (1:9) and administered orally (20 µg per day) to pups from postnatal day (P) 1–5. Adult deletion was accomplished by administering tamoxifen orally (1 mg per day) for 5 consecutive days beginning at P21, then one week thereafter. Wild-type littermates (Pdpnfl/2) treated with the same regimen were used as controls. Mice deficient for Pdpn in pericytes/fibroblasts including FRCs (Pdpnfl/2/PdgfraCre) or in endothelial cells (Pdpnfl/2;Tie2Cre) were generated by crossing Pdpnfl/2 mice with PdgfraCre Tg mice (Tg[Pdgfra-cre9Rha] or Tie2Cre Tg mice (Tg[Tdk-cre]1Ywa) respectively.

Conditional Clec-2 knockout mice were generated in which exons 3 and 4 of the Clec-2 allele were flanked by loxP sites (Clec-2fl/2). Deletion of exons 3 and 4 induces a premature stop codon that blocks the expression of the extracellular domain of CLEC-2 (Supplementary Fig. 8a). ES clones with correct homologous recombination were microinjected into B6 blastocysts, which were subsequently implanted into pseudopregnant foster mothers. Male chimaeras were bred with ACTB-FLPe females (B6.Cg-Tg(ACTFLP1c9205Dym/J, Jackson Laboratory) for germline transmission and removal of the Neo cassette. Heterozygous mice were then crossed to generate Clec-2fl/2 mice. Clec-2fl/2 mice were crossed with PdgfbrCre Tg mice (C57BL/6-Tg[Pdgfrb-cre9Rha]) to generate mice deficient for Clec-2 on platelets (Clec-2fl/2,PdgfbrCre mice).

To determine the vascular permeability in MLNs, PDPN expression on LN cells was analysed by flow cytometry. Briefly, LN were digested in digestion buffer (RPMI-1640 (Invitrogen) containing 0.2 mg ml−1 collagenase P (Roche), 0.1 mg ml−1 DNase I (Invitrogen) and 0.8 mg ml−1 dispase (Roche)) at 37 °C for 20 min. Digested LNs were vigorously mixed to ensure disruption of capsule and release of leukocytes. Cell suspensions were placed in ice-cold PBS containing 2% FCS and 5 mM EDTA and centrifuged at 300 × g for 10 min at 4 °C. Pellets were resuspended in digestion buffer. Washed cells (5 × 10^6) were blocked for 15 min on ice (in HBSS containing Ca^2+ and Mg^2+, 2% horse serum, 20 µg ml−1 anti-CD16/CD32 (clone 2.4G2, BD Pharmingen)). Isolated cells were incubated with antibodies to PDPN (clone 8.1.1) and biotinylated CD31 (clone MEC13.3, BD Pharmingen) for 30 min on ice and washed. Cells were then incubated with fluorescent dye-conjugated antibodies: AF488 conjugated goat anti-Syrian hamster IgG (Jackson Immunoresearch), PerCP-conjugated streptavidin (BioLegend) and PE-conjugated rat anti-mouse CD45 (clone 30-F11, BD Pharmingen) for 20 min on ice. Flow cytometry analysis was performed on a FACSCalibur (Becton Dickinson). Postnatal CLEC-2 deletion. Wild-type and Rag1−/− mice were intraperitoneally injected (i.p.) injected with 8 µg per gram of body weight of a monoclonal antibody to CLEC-2 (clone INU1)14 at P1, P6 and P11. These mice were then killed on P15.

Labelling of erythrocytes or platelets. For erythrocyte labelling, whole blood from wild-type mice was collected into EDTA-coated Microvette 500 K3E tubes (Sarstedt), washed in HBSS (without Ca^2+ and Mg^2+), centrifuged at 300 × g for 5 min at 4 °C, and resuspended in HBSS (without Ca^2+ and Mg^2+) at a 1:5 dilution of the original volume and incubated with 5 µM CM-Dil (Invitrogen). Washed, labelled RBCs were resuspended at 75% haematocrit and 200 µl were intravenously injected into mice. For platelet labelling, platelet-rich plasma was intravenously injected into mice. Labelled platelet, platelet-rich plasma was obtained by collecting whole blood into an Eppendorf tube containing 20 U heparin, adding 500 µl modified Tyrode’s buffer (137 mM NaCl, 0.3 mM Na2HPO4, 2 mM KCl, 12 mM NaHCO3, 5 mM HEPES, 5 mM glucose, pH 7.3) containing 0.35% BSA and centrifuged for 8 min at 1000 g at room temperature. Platelets were collected by centrifuging for 10 min at 1000 g. Washed platelets were incubated with 5 µM CellTracker Green (Invitrogen) for 30 min at 37 °C. Labelled Platelets were washed in HBSS (without Ca^2+ and Mg^2+) and resuspended in HBSS (without Ca^2+ and Mg^2+) at a 1:5 dilution of the original volume and incubated with an anti-CD31 antibody and analysed for the presence of fluorescently labelled RBCs outside the blood vessels. Evans blue dye (2% in saline) was intravenously injected 5 min before killing the mice. Mice were perfused with heparinized saline and LNs were placed in formamide (Fisher Scientific) overnight at 55 °C. Tissues were removed from formamide and absorbance was measured at 620 nm in a spectrophotometer20.

Vascular permeability analysis. To determine the vascular permeability in MLNs, 250 µg of lysine-fixable FITC-dextran (2,000 kDa, Invitrogen) in 40 µl total volume physiological saline was injected immediately before killing animals. Frozen sections of MLNs were stained with anti-CD31 or PNA antibodies and analysed for the presence of dextran outside the blood vessels. Alternatively, 500 µg of lysine-fixable FITC-dextran (2,000 kDa, Invitrogen) or fluorescein labelled RBCs resuspended at a 75% haematocrit were intravenously injected into mice 1 min or 10 min, respectively, before intravenous injection of dextran. Efficiency of the dextran was confirmed using an anti-CD31 antibody and analysed for the presence of fluorescein labelled RBCs outside the blood vessels. Evans blue dye (2% in saline) was intravenously injected 5 min before killing the mice. Mice were perfused with heparinized saline and LNs were placed in formamide (Fisher Scientific) overnight at 55 °C. Tissues were removed from formamide and absorbance was measured at 620 nm in a spectrophotometer20.

Transmission electron microscopy (TEM). LNs were removed and fixed overnight at 4 °C in 2% gluteraldehyde in 0.1 M cacodylate buffer (pH7.3). Tissues were washed in 0.1 M cacodylate buffer and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. Tissues were dehydrated through graded alcohols and embedded in EPO resin (EMS). Thin sections (~300 nm) were cut using an ultramicrotome (RMC 7000, RMC) equipped with a diamond knife and stained with toluidine blue, some of which were imaged. Ultrathin sections (~70 nm) were stained with uranyl acetate and lead citrate and visualized using a...
Hitachi H-7600 electron microscope with a 4 megapixel digital monochrome camera and AMT-EM image acquisition software (Advanced Microscopy Techniques).

Platelet transfusion experiments. Platelets were isolated as described above and were intravenously injected into Clec-2/−/− BM chimaeras once a day for 4 consecutive days. For S1P+/− mice, wild-type or Clec-2/−/− platelets were isolated as before and resuspended in HBSS without Ca2+ and Mg2+. Either wild-type or Clec-2/−/− platelets were administered once a day for 4 consecutive days. One minute before euthanizing, mice were injected (i.v.) with 500 µg of FITC-Dextran (2,000 kDa, Invitrogen) in 100 µl HBSS without Ca2+ and Mg2+. Either wild-type or Clec-2/−/− platelets were administered once a day for 4 consecutive days. One minute before euthanizing, mice were injected (i.v.) with 500 µg of FITC-Dextran (2,000 kDa, Invitrogen) in 100 µl HBSS without Ca2+ and Mg2+.

Immune challenge. Three- to four-week-old mice received subcutaneous injections of 250 µg ovalbumin/complete Freund’s adjuvant (OVA/CA, Sigma-Aldrich) into the hindlimb. Control lateral hindlimbs were injected with an equivalent volume of saline as a control. Mice were killed 1 week after challenge and popliteal LNs were processed for immunofluorescence staining.

**L-selectin blockade and in vitro leukocyte rolling assay.** Three- to four-week-old mice were challenged with 250 µg OVA/CA into the hindlimb. Mice were then administered i.p. 30 µg of an anti-mouse L-selectin antibody (Mel-14) every other day for 1 week. Mice were killed and popliteal LNs were dissected and analysed.

To determine the blocking efficiency of Mel-14, an in vitro lymphocyte rolling assay was used. Briefly, 100 µg ml−1 streptavidin was coated on 35-mm polystyrene dishes overnight at 4°C. After washing three times with HBSS, the dishes were blocked with 1% human serum albumin in HBSS for 2 h at 4°C and then incubated with biotin conjugated 6-sulfo-sLex, a ligand for L-selectin, for 2 h at 4°C. Isolation of peripheral leukocytes was described previously36. Heparanized blood was obtained from wild-type or Pdpn−/−CagCre mice that were treated with Mel-14 or rat IgG. After lysis of red blood cells, leukocytes were centrifuged at 100g for 10 min. Leukocytes (0.1 × 106 per ml) were perfused over 6-sulfo-sLex coated surface under shear stress at 1.0 dyn per cm2. The number of rolling cells was counted using the Element software (Nikon).

**In vivo blockade of VE-cad and integrin αIIbβ3.** After hindlimb challenge with OVA/CA, mice were i.p. injected with 30 µg every other day of a monoclonal antibody against VE-cad (clone 11D4.1, BD Pharmingen) or against activated integrin αIIbβ3 (clone JON/1)37. One week after immune challenge, Evans blue dye permeability assays were performed as described above.

**Peripheral lymphocyte counts.** For measuring peripheral lymphocyte counts, whole blood obtained in EDTA-coated tubes was used to obtain a complete blood count using a Hemavet.

**Development of PDPN+/− and PDPN−/− cell line.** Parental murine melanoma cell line B16-F0, which contains PDPN+ and PDPN− cells, was purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM containing 10% heat-inactivated FBS and 1% t-glutamine/penicillin/streptomycin (Cellgro) at 37°C in a humidified atmosphere of 5% CO2. B16-F0 cells were stained with anti-murine PDPN monoclonal antibody (clone 8.1.1) and a DyLight488-conjugated secondary antibody. Stained cells were then sorted with a FACSAria III cell sorter (BD Biosciences) to generate the PDPN+ and PDPN− B16-F0 melanoma cells.

**Analysis of S1P concentration.** Platelets were isolated from wild-type or Clec-2−/− mice as before. After being washed with modified Tyrode’s buffer containing 0.35% fatty acid-free BSA (Sigma-Aldrich), platelets (1 × 108 in 100 µl) were then incubated with a monoclonal antibody against CLEC-2 (clone INU1 at 10 µg ml−1) for 10 min at room temperature. The supernatant was collected and S1P concentration was determined using an S1P ELISA kit (Echelon Biosciences) according to the manufacturer’s protocol. Alternatively, platelets were incubated with 5 × 105 PDPN+ or PDPN− cells for 10 min at room temperature. Then the supernatant was collected and S1P concentration was quantitated by electrospray ionisation tandem mass spectrometry (ESI-MS/MS) using stable isotope dilution with heptadecuterated S1P (Avanti Polar Lipids) as the internal standard as described previously38.

**Ex vivo LN slice culture.** MLNs were dissected from wild-type and Pdpn−/- deficient mice and embedded in low melting point agarose (Invtrogen) at 37°C. Blocks were cooled on ice and sectioned (~250 µm) using a Vibratome (McIlwain tissue chopper). LN slices were then co-incubated with washed platelets (2 × 107 in 100 µl of DMEM containing 10% charcoal stripped FBS; Invitrogen), isolated from wild-type, Clec-2−/−, or S1P+/− mice, for 1.5 h at 37°C with gentle shaking. In some experiments, normal FBS or the S1PR1 antagonist, W146 (5 µM, Cayman Chemical), was included. MLN slices were then fixed in 4% PFA in PBS for 30 min and processed for cryopreservation. Cryosections were stained with antibodies against CD31 (clone ZH8, Abcam) and VE-cad (clone 11D4.1, BD Pharmingen).

**Quantification of Pdpn deletion, dextran leakage or VE-cad intensity.** PDPN levels were calculated using Imagej software to compare the mean intensities between the groups from a minimum of 5 low magnification (×10) images. For quantification of FITC–dextran leakage, six cryosections were cut per mouse and each section was analysed for the total number of HEVs (~50 per section of MLNs) and non-HEVs based on CD31 or PNA staining and morphology. The number of vessels that exhibited dextran outside the vessel was determined and the percentage of ‘leaky’ vessels compared to the total number of that vessel type was determined. Similarly, the percentage of VE-cad expression on vessels from MLN cryosections was determined by comparing the number of HEV and non-HEV that were VE-cad positive to the total number of vessel type. Alternatively, VE-cad intensities on HEVs were determined using Imagej analysis software.

**Statistical analysis.** Statistical tests were performed using Prism software (GraphPad). Two-sided, Student’s t-tests were performed after the data were confirmed to fulfill the criteria of normal distribution and equal variance. Differences were considered statistically significant when P < 0.05.

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