The CLEC-2–podoplanin axis controls the contractility of fibroblastic reticular cells and lymph node microarchitecture

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Lymph nodes (LNs) are highly structured organs that support immune responses and control unwanted immunological activation1. LNs increase vastly in size during immune responses and then must contract again upon resolution. Although lymphocyte proliferation and vascular remodeling contribute to this swelling2–4, the contributions of the reticular network have not been understood. Here we demonstrate that podoplanin (PDPN) regulates actomyosin contractility in FRCs. Under resting conditions, when FRCs are unlikely to encounter mature DCs expressing the PDPN receptor CLEC-2, PDPN endowed FRCs with contractile function and exerted tension within the reticulum. Upon inflammation, CLEC-2 on mature DCs potently attenuated PDPN-mediated contractility, which resulted in FRC relaxation and reduced tissue stiffness. Disrupting PDPN function altered the homeostasis and spacing of FRCs and T cells, which resulted in an expanded reticular network and enhanced immunity.

In lymph nodes, fibroblastic reticular cells (FRCs) form a collagen-based reticular network that supports migratory dendritic cells (DCs) and T cells and transports lymph. A hallmark of FRCs is their propensity to contract collagen, yet this function is poorly understood. Here we demonstrate that podoplanin (PDPN) regulates actomyosin contractility in FRCs. Under resting conditions, when FRCs are unlikely to encounter mature DCs expressing the PDPN receptor CLEC-2, PDPN endowed FRCs with contractile function and exerted tension within the reticulum. Upon inflammation, CLEC-2 on mature DCs potently attenuated PDPN-mediated contractility, which resulted in FRC relaxation and reduced tissue stiffness. Disrupting PDPN function altered the homeostasis and spacing of FRCs and T cells, which resulted in an expanded reticular network and enhanced immunity.
In sum, our results identify PDPN as a master regulator of actomyosin contractility in FRCs. PDPN signaling maintained FRCs in a highly contracted state in healthy, resting organs. Upon an inflammatory response, the interaction between migratory DCs and FRCs allowed CLEC-2 to block PDPN, thereby attenuating contractility and relaxing the reticulum. Consequently, these microanatomical changes allowed the LN to increase in size and meet the spatial demands of the expanding lymphocyte pool.

RESULTS

Regulation of FRC actomyosin contractility by PDPN

To investigate the function of PDPN in LN FRCs, we initially isolated FRCs from wild-type mice and PDPN-deficient (Pdpn–/–) mice. While Pdpn–/– mice generally die soon after birth due to blood-lymph mixing and edema, when crossed onto a C57BL/6 background, approximately 20% of the mice thrive, and these served as a source of Pdpn–/– FRCs for our study14. FRCs from Pdpn–/– mice expressed normal amounts of YAP, a transcription factor reported to be activated by mechanical forces in diverse cell types15,16 and in our studies (Fig. 1c). We next sought to determine the mechanism underlying PDPN deficiency in the FRC cytoskeleton in collagen-based three-dimensional deformable matrices, which more closely simulate the LN microenvironment18. PDPN-deficient FRCs were elongated and extended F-actin-rich membrane protrusions (Fig. 1a,b). Such protrusions were less abundant on Pdpn–/– FRCs than on wild-type FRCs; however, each protrusion extending from the cell body of Pdpn–/– FRCs was much longer than those of wild-type FRCs (Fig. 1c,d).

Finally, as in two dimensions, PDPN-deficient FRCs covered a smaller area in three dimensions than did wild-type FRCs (Fig. 1e).

Given that adhesion and spreading are related to cell contraction, we next used multiple approaches to investigate whether PDPN influences FRC contractility. First, we treated FRCs with Y27632, an inhibitor of the kinase ROCK, a master regulator of contractility24. Y27632-treated FRCs exhibited extensive cell elongation similar to that of Pdpn–/– FRCs; however, Y27632-treated FRCs exhibited more protrusions than did Pdpn–/– FRCs (Supplementary Fig. 1d–f), which suggested that PDPN might signal through additional pathways. Next we directly assessed contractility by examining the contraction of three-dimensional collagen gels. Wild-type FRCs were much more efficient at contracting collagen than were Pdpn–/– FRCs (Fig. 1f). Finally, a third measure of contractility, we assessed the abundance of nuclear (active) YAP, a transcription factor reported to control contractility and promote the tumor-supporting functions of cancer-associated fibroblasts25. In agreement with the data obtained for collagen contraction, Pdpn–/– FRCs had significantly less nuclear YAP than did wild-type FRCs (Fig. 1g and Supplementary Fig. 1g). Together these results indicated that PDPN was a critical regulator of adhesion, elongation, and contraction in FRCs.

Molecular regulation of PDPN-mediated contractility

Next we sought to determine the mechanism underlying PDPN functions in FRCs. The only known proximal signaling mechanism by which PDPN might exert any direct effect on the cytoskeleton is through binding to and activating proteins of the ezrin, radixin, and moesin (ERM) family, which link membrane proteins to the cytoskeleton. In overexpression studies, PDPN was found to interact with ERM proteins through a cluster of basic residues in the cytoplasmic...
The PDPN cytoplasmic tail controls FRC elongation but is dispensable for contraction.

(a) Confocal microscopy of wild-type and Δcyto FRCs seeded into three-dimensional gels (numbers as in Fig. 1a). Scale bars, 100 μm (left) or 20 μm (right). (b) Morphology index of wild-type, Pdpn−/− and Δcyto FRCs. (c,d) Quantification of protrusions (c) and length of protrusions (d) of wild-type, Pdpn−/− and Δcyto FRCs. (e) Contraction of collagen gels by Pdpn−/− and Δcyto FRCs, relative to that induced by wild-type FRCs. (f) Frequency of nuclear YAP in wild-type, Pdpn−/− and Δcyto FRCs. Each symbol (b–f) represents an individual cell; small horizontal lines indicate the mean (± s.d.). NS, not significant; *P < 0.01 and **P < 0.001 (Mann-Whitney test (b–f) or one-sample t-test (e)). Data are representative of three independent experiments (a–d; mean ± s.d. of n > 50 cells in each in b–d), three to eight independent experiments (e; mean ± s.d.) or two independent experiments (f; n > 9 cells per experiment).

Control of the proliferation and survival of FRCs by PDPN

Stromal fibroblasts are exquisitely sensitive to the stiffness of the extracellular matrix they adhere to, which in turn affects their cytoskeletal contraction. Greater substrate stiffness results in tension in fibroblasts, which can affect their growth and survival. Thus, we investigated whether the attenuated contractility in Pdpn−/− FRCs affected their proliferation or survival. Whereas the number of wild-type and Δcyto FRCs remained steady over a 4-day period, Pdpn−/− FRCs increased up to fourfold in number (Fig. 4a). Pdpn−/− FRCs proliferated more extensively than did wild-type FRCs (Fig. 4b,c) and survived better than either wild-type FRCs or Δcyto FRCs (Fig. 4d,e). Overall, these data demonstrated the involvement of PDPN signaling in regulating the growth and survival of FRCs.

Figure 2 The PDPN cytoplasmic tail controls FRC elongation but is dispensable for contraction.

(a) Confocal microscopy of wild-type and Δcyto FRCs seeded into three-dimensional gels (numbers as in Fig. 1a). Scale bars, 100 μm (left) or 20 μm (right). (b) Morphology index of wild-type, Pdpn−/− and Δcyto FRCs. (c,d) Quantification of protrusions (c) and length of protrusions (d) of wild-type, Pdpn−/− and Δcyto FRCs. (e) Contraction of collagen gels by Pdpn−/− and Δcyto FRCs, relative to that induced by wild-type FRCs. (f) Frequency of nuclear YAP in wild-type, Pdpn−/− and Δcyto FRCs. Each symbol (b–f) represents an individual cell; small horizontal lines indicate the mean (± s.d.). NS, not significant; *P < 0.01 and **P < 0.001 (Mann-Whitney test (b–f) or one-sample t-test (e)). Data are representative of three independent experiments (a–d; mean ± s.d. of n > 50 cells in each in b–d), three to eight independent experiments (e; mean ± s.d.) or two independent experiments (f; n > 9 cells per experiment).
Control of LN stiffness and FRC proliferation by PDVPN

Given the effects of PDVPN deficiency on the FRC cytoskeleton and actomyosin contractility on a cellular level, we next assessed the effect of blocking PDVPN on the FRC network in vivo. Due to the reduced viability of mice with global PDVPN deficiency (Pdpn−/− mice), we used a PDPN-blocking monoclonal antibody (clone 8.1.1) to recapitulate both the elongation and impaired contraction observed in Pdpn−/− FRCs (Supplementary Fig. 4a,b). We injected 8.1.1 intravenously into wild-type mice and examined their LNs by flow cytometry. We observed no gross abnormalities in mice treated with anti-PDVPN, such as the bleeding reported in some transgenic mice19,21, inflammation, or major disorganization of lymphocytes (Supplementary Fig. 4c–g and data not shown). However, the brachial LNs of wild-type mice treated with anti-PDVPN were significantly larger than those of mice treated with the isotype-matched control antibody, and while LNs from wild-type mice treated with

Figure 3 PDVPN signals through ERM and MLC to control contraction. (a) Microscopy of staining for phosphorylated (p-) ezrin in wild-type, Pdpn−/− and Δcyto FRCs. Scale bar, 10 μm. (b) Immunoblot analysis of phosphorylated and total ERM in wild-type, Pdpn−/− and Δcyto FRCs. Scale bar, 10 μm. (c) Microscopy of staining for phosphorylated MLC in wild-type, Pdpn−/− and Δcyto FRCs. Scale bar, 10 μm. (d) Immunoblot analysis of phosphorylated and total MLC in wild-type, Pdpn−/− and Δcyto FRCs. (e) Immunoblot analysis of active RhoA (RhoA-GTP) and total RhoA in wild-type, Pdpn−/− and Δcyto FRCs. Numbers below lanes (b,d,e) indicate band density for the activated protein relative to total protein, presented relative to that ratio in wild-type cells. Data are representative of three to five independent experiments.

Figure 4 PDVPN maintains normal proliferation and survival of FRCs. (a) Quantification of cells present over 4 d of culture, as measured by ATP content, presented relative to results for wild-type cells at day 1, set as 1. (b,c) CFSE content in wild-type, Pdpn−/− and Δcyto FRCs after 48 h of culture (b) or 96 h of culture (c). Numbers above lines (left) indicate mean fluorescence intensity. (d) Flow cytometry of wild-type, Pdpn−/− and Δcyto FRCs stained for annexin V and with the membrane-impermeable DNA-intercalating dye 7-amino-actinomycin D (7-AAD). Numbers in outlined areas indicate percent annexin V–positive (and 7-amino-actinomycin D–negative) cells. (e) Quantification of annexin V–positive (AnnV+) wild-type, Pdpn−/− and Δcyto FRCs (as in d). Each symbol represents an individual population of cells from one mouse; small horizontal lines indicate the mean ± s.d. *P < 0.05 (Student’s t-test (a) or Mann-Whitney test (e)). Data are representative of three independent experiments (a, mean ± s.d. of three wells per experiment), two independent experiments (b,c) or four independent experiments (d) or are from four independent experiments (e).
anti-PDPN had slightly greater cellularity than those from mice treated with an isotype-matched control antibody, this was not concomitant with the enhanced mass (Fig. 5a,b). These findings led us to question whether the stiffness of the LN was altered. Using a standard compression test, we found that LNs from wild-type mice were significantly less stiff and more deformable following antibody blockade of PDPN than after treatment with an isotype-matched control antibody (Fig. 5c).

Next we sought to determine whether FRCs would respond to the relaxation noted above by increasing in number as they did in vitro. Two days after the administration of antibody to wild-type mice, the number of FRCs that had incorporated the thymidine analog BrdU (proliferating FRCs) and, accordingly, the total number of FRCs were significantly greater in mice treated with monoclonal antibody 8.1.1 than in their counterparts treated with an isotype-matched control antibody (Fig. 5d,e). Additionally, FRCs in mice treated with anti-PDPN were much larger than those in mice treated with an isotype-matched control antibody (Supplementary Fig. 4h,i), consistent with a blasting phenotype.

We also assessed the effect of PDPN deficiency on FRCs with Pdpnfl/fl Pdgfrb-Cre mice, in which loxP-flanked Pdpn alleles are deleted by Cre recombine expressed from the gene encoding the receptor for platelet-derived growth factor-β (Pdgfrb) and thus their FRCs lack PDPN19. Consistent with results obtained for mice treated with anti-PDPN, Pdpnfl/flPdgfrb-Cre mice exhibited larger LNs with greater total cellularity and a greater number of FRCs than did Pdpnfl/fl control mice (without expression of Pdgfrb-Cre) (Supplementary Fig. 5a–c). However, we did not pursue additional studies of Pdpnfl/flPdgfrb-Cre mice due to their phenotype of bleeding related to a disruption in the integrity of high endothelial venules (HEVs) that has been previously reported19 (Supplementary Fig. 5d).

Finally, we observed no difference between Δcyto mice and wild-type mice in LN mass, total cellularity, or number of FRCs (Fig. 5f–h). Additionally, we did not observe bleeding around HEVs in Δcyto mice (Supplementary Fig. 5e). Given that FRCs cultured from these mice exhibited an elongation phenotype but normal contractility, these in vivo results indicated that FRC contractility was critical for maintaining normal LN size and number of FRCs.
Expansion of the FRC network upon blockade of PDPN

Next we sought to define the role of PDPN in the LN FRC network. First, we investigated conduit function by injecting the tracer fluorescein isothiocyanate into the footpads of mice treated with isotype-matched control antibody or anti-PDPN and collecting the popliteal LNs 4 h later. We noted no obvious abnormalities in access of the tracer to conduits in either the paracortex or cortical regions beneath the subcapsular sinus in mice treated with anti-PDPN (Supplementary Fig. 5f).

Next we investigated the effect of PDPN blockade on the structure of the FRC network. For this we used Rosa26-eYFP<sup>fl/fl</sup>/Ccl19-Cre mice, in which Cre expressed by the locus encoding the chemokine CCL19 drives the excision of a transcriptional stop sequence before the gene encoding enhanced yellow fluorescent protein (eYFP) inserted into the ubiquitous Rosa26 locus, which results in expression of eYFP only in FRCs<sup>39</sup>. We treated these mice with anti-PDPN or an isotype-matched control antibody. Confocal microscopy combined with three-dimensional reconstruction and isosurface rendering revealed that the network had a finer appearance with more space between fibers in Rosa26-eYFP<sup>fl/fl</sup>/Ccl19-Cre mice treated with anti-PDPN than in those treated with the isotype-matched control antibody (Fig. 5i). Quantitative analysis confirmed that the surface area of FRCs and an extracellular matrix component they secrete, ER-T.R7, was lower in LNs from mice treated with anti-PDPN than in those from mice treated with isotype-matched control antibody (Fig. 5j–k). We observed changes to the FRC network in Pdpn<sup>−/−</sup>/Pdgfrb<sup>Cre</sup> Cre mice that were similar to those in mice treated with anti-PDPN (Supplementary Fig. 5g–i). Finally, and consistent with our observations that LN mass and FRC numbers remained unchanged upon deletion of the PDPN cytoplasmic tail, the network structure in A<sup>cyto</sup>&nbsp;LNs was similar to that in wild-type LNs (Fig. 5l–n).

To determine whether FRCs were further apart from each other, we analyzed nuclear distance by measuring the distance to the closest neighboring FRC nucleus. FRCs were closer to each other in LNs of mice treated with isotype-matched control antibody than in those of mice treated with anti-PDPN (Fig. 5o and Supplementary Fig. 5j,k). Thus, the stromal relaxation that occurred upon attenuation of PDPN-mediated contractility led to LN enlargement and expansion of the FRC network.

Restraint of T cell responses by PDPN-mediated contractility

We next addressed how blocking PDPN function and FRC contractility would affect an immune response. For this, we treated wild-type mice with anti-PDPN or an isotype-matched control antibody, followed 2 d later by injection of OT-I CD8<sup>+</sup> T cells (which have transgenic expression of an ovalbumin (OVA)-specific T cell antigen receptor) labeled with the cytosolic dye CFSE and immunization with lipopolysaccharide (LPS) and OVA. Notably, the PDPN-specific antibody did not block binding of CLEC-2–Fc (CLEC-2 linked to a rabbit Fc fragment) to FRCs or affect DC numbers in steady-state or inflammatory conditions (Supplementary Fig. 6a–c). At 96 h after immunization with LPS and OVA, we found a significantly greater number of OT-I T cells in LNs treated with anti-PDPN than in those treated with an isotype-matched control antibody (Fig. 6a). Accordingly, at 48 h after immunization, divided OT-I T cells were more abundant in LNs treated with anti-PDPN than in those treated with the isotype-matched control antibody (Fig. 6b). FRCs suppress the proliferation of activated T cells through cell-cell contact<sup>10–33</sup>. Given that we observed greater spacing between FRCs after blockade of PDPN, we investigated whether there was a change in the number of T cells touching FRCs in the mice treated with anti-PDPN. Indeed, fewer T cells were in contact with the FRC network after blockade of PDPN than after treatment with the isotype-matched control antibody (Fig. 6c); this may have contributed to the increased T cell response. Overall, these findings indicated that PDPN-mediated control of FRC contractility was critical for restraining LN swelling and T cell responses.

Inhibition of FRC contraction by CLEC-2 engagement of PDPN

PDPN<sup>−/−</sup> FRCs come into contact with CLEC-2 on migratory DCs and platelets<sup>18,19,21</sup>, which raises the question of how binding of CLEC-2 to PDPN affects FRCs. To determine if CLEC-2 transmits a signal to FRCs, we cultured wild-type FRCs in three-dimensional matrices and incubated them with soluble CLEC-2–Fc or, as a control, rabbit immunoglobulin G (IgG). Within 3 h, the FRCs became elongated, and by 12 h, they closely resembled Pdpn<sup>−/−</sup> FRCs (Fig. 7a,b). CLEC-2–Fc or rabbit IgG had no effect on the elongation of Pdpn<sup>−/−</sup> or A<sup>cyto</sup>&nbsp;FRCs (Supplementary Fig. 7a), as expected, because FRCs do not express Fc receptors<sup>4</sup>. In addition, treatment with CLEC-2–Fc impaired the spreading of FRCs on collagen (Supplementary Fig. 7b). To determine whether the same effect could be achieved with CLEC-2 naturally expressed by cells, we cultured wild-type FRCs together with LPS-activated wild-type or CLEC-2-deficient (Clec1b<sup>−/−</sup>) bone marrow–derived DCs (BMDCs) or with wild-type or Clec1b<sup>−/−</sup> platelets. Notably, CLEC-2<sup>+</sup> DCs and platelets induced elongation of FRCs to a degree similar to that induced by soluble CLEC-2–Fc (Fig. 7c,d). CLEC-2–Fc also induced clustering of PDPN and the formation of PDPN-rich protrusions (Fig. 7e,f). Thus, we investigated whether this elongation was dependent on actin polymerization. Indeed, treatment with cytochalasin D at a concentration that preserved cytoskeletal integrity significantly diminished the elongation of FRCs elicited by CLEC-2–Fc (P = 0.0117; Supplementary Fig. 7c).

The finding that CLEC-2 had the same effect on FRCs as PDPN deficiency had prompted us to assess whether binding of CLEC-2 inhibited contractility. The binding of CLEC-2 to wild-type and A<sup>cyto</sup>&nbsp;FRCs caused a significant reduction in collagen contraction and the
Having shown that antibody blockade of PDPN led to increased LN swelling in the steady state and T cell proliferation following immunization and that CLEC-2 inhibited PDPN, we sought to determine whether a CLEC-2 signal from DCs was necessary and sufficient to cause LN swelling. For this, we injected wild-type and Clec1b<sup>−/−</sup> BMDCs into the footpads of wild-type mice and measured LN mass 1, 2, and 5 d later. We found that injection of wild-type BMDCs significantly increased LN mass, as previously described<sup>13</sup>, but injection of Clec1b<sup>−/−</sup> BMDCs led to significantly less LN swelling in mice that received Clec1b<sup>−/−</sup> BMDCs than in those that received wild-type BMDCs, at each time point examined (Fig. 8a). However, the impaired recruitment of CLEC-2-null cells is transient, and the number of wild-type DCs and Clec1b<sup>−/−</sup> DCs in draining LNs is no longer different as early as 3 d after injection<sup>18</sup>. Therefore, the finding that LN mass remained significantly lower 5 d after DC injection, when equal numbers of wild-type and Clec1b<sup>−/−</sup> migratory DCs are present in LNs, should be interpreted as an absence of CLEC-2 signaling, rather than impaired DC recruitment. This notion supports our hypothesis that an inhibitory signal from CLEC-2 on migratory DCs is required for LN expansion.

Next we hypothesized that a lack of CLEC-2 during an immune response would prevent the expansion of LNs and T cell proliferation.
Thus, to investigate the necessity of CLEC-2 on endogenous DCs, we immunized Clec1b<sup>fl/fl</sup> Cd11c<sup>-Cre</sup> mice (which have loxp-flanked Clec1b alleles deleted by Cre expressed from the gene encoding the integrin CD11c (Igax-Cre; called 'Cd11c-Cre here)) and Clec1b<sup>fl/fl</sup> mice (which do not express Cd11c-Cre) with LPS and collected their LNs 24 h later. In agreement with published data<sup>18,34</sup>, we observed a dampened immune response, with smaller LNs and less total LN cellularity in Clec1b<sup>fl/fl</sup> Cd11c-Cre mice than in Clec1b<sup>fl/fl</sup> mice (Fig. 8b,c). As expected, this observation was due to the decreased number of migratory DCs entering into the LNs and the dampened T cell response in Clec1b<sup>fl/fl</sup> Cd11c-Cre mice relative to that in Clec1b<sup>fl/fl</sup> mice (Fig. 8d,e).

Next we sought to determine whether a lack of CLEC-2 would impair expansion of the FRC network. While FRC numbers increased following immunization with LPS, this increase was dampened when the CLEC-2 signal from DCs was absent (Fig. 8f). Furthermore, as we had observed in mice treated with anti-PDPN (Fig. 5i), the FRC network significantly expanded in Clec1b<sup>fl/fl</sup> control mice upon immunization (Fig. 8g–i). Notably, while there was no significant difference between PBS-treated Clec1b<sup>fl/fl</sup> mice and PBS-treated Clec1b<sup>fl/fl</sup> Cd11c-Cre mice in their FRC networks, the LPS-induced expansion of the network was abolished in Clec1b<sup>fl/fl</sup> Cd11c-Cre mice (Fig. 8g–i). These results confirmed published findings that a lack of CLEC-2 impairs the arrival of DCs in draining LNs and dampens immune responses<sup>18</sup>. Furthermore, we advanced those findings by demonstrating that the FRC network expanded to accommodate increasing LN size and that this expansion required a CLEC-2 signal from DCs. While in this setting the effect CLEC-2 deficiency cannot be separated from that of impaired DC migration, these results nevertheless supported our findings obtained with the PDPN-specific antibody.

As a final assessment of whether CLEC-2 itself delivers a signal to relax the FRC network, we injected CLEC-2–Fc intravenously into wild-type mice and examined LNs at 1 h and 4 h after injection. Consistent with our hypothesis, after only 1 h, LNs of mice treated with CLEC-2–Fc were significantly larger than those of mice treated with rabbit IgG and, at 4 h, the LNs had already returned nearly to their baseline weight (Fig. 8). This finding was not due to an influx of cells or inflammation (Supplementary Fig. 7h–l). LECs also express PDPN, which raises the possibility that the CLEC-2 signal might alter vascular integrity. However, while Pdpn<sup>−/−</sup> LECs were elongated compared with wild-type LECs, unlike FRCs, wild-type LECs did not respond to soluble CLEC-2 by elongating (Supplementary Fig. 7m). Furthermore, injection of anti-PDPN did not lead to changes in lymphatic pulsatile activity at early (1 h) or late (48 h) time points (data not shown), which suggested that the in vivo effects of PDPN blockade were not attributable to global alterations in lymph flow. However, in LNs treated with CLEC-2–Fc, FRCs were disorganized, particularly around HEVs (Supplementary Fig. 7n). Thus, CLEC-2–Fc–induced relaxation of FRCs may alter HEV integrity and allow blood or plasma to enter LNs.
Collectively, these data indicated that CLEC-2 inhibited PDPN function and caused FRCs to relax. This relaxation led to increased spacing in the paracortical reticular network and allowed LNs to expand. However, upon attenuation of the CLEC-2 signal, FRCs regained their contractile ability, which restored normal LN size and microarchitecture (Supplementary Fig. 8).

**DISCUSSION**

Here we have identified PDPN as a master regulator of the contractile force generated by FRCs and have elucidated a critical function for FRC contractility in maintaining LN microarchitecture. Under non-inflammatory conditions, PDPN maintained FRCs in a highly contracted state. Disruption of PDPN-mediated contractility caused LN enlargement in part via relaxation and expansion of the FRC network. Unexpectedly, these changes did not affect conduit permeability, in line with a published report demonstrating prolonged conduit function following ablation of FRCs.

Notably, we discovered that CLEC-2 inhibited PDPN signaling in FRCs. Published studies of the PDPN–CLEC-2 interaction have focused on signaling downstream of CLEC-2 (refs. 18,19,21). Here we have demonstrated that signaling also occurred downstream of PDPN and that its engagement of CLEC-2 on DCs diminished FRC contractility. CLEC-2 demonstrated that signaling also occurred downstream of PDPN and that its extracellular or transmembrane domain of PDPN is required for its localization to lipid rafts40, where the affinity of the binding of CLEC-2 to PDPN is 24.5 μM (ref. 16), whereas a high-affinity antibody can be in the nanomolar or even picomolar range39. Furthermore, within minutes of engaging soluble PDPN, CLEC-2 is internalized18. Thus, the inhibition of PDPN caused by migratory DCs would probably be quickly relieved.

Our finding that the cytoplasmic tail of PDPN was dispensable for FRC contractility was unexpected and suggested that its extracellular or transmembrane domains mediate this hallmark function. Indeed, the interaction of PDPN with CD44 and galectin-8 regulates cell migration and adhesion57,38. Furthermore, PDPN interacts with CD9, a tetraspanin that modulates integrin signaling59. Furthermore, the transmembrane domain of PDPN is required for its localization to lipid rafts60, where CD44 is also retained upon the differentiation of fibroblasts into myo-fibroblasts mediated by transforming growth factor-β41. Additionally, our findings obtained with Δcyto FRCs indicating that the PDPN cytoplasmic tail was required for FRC elongation despite the finding that Δcyto FRCs had normal levels of activated ERM proteins suggested that the cytoplasmic tail has other signaling mechanisms. In fact, two serine residues in the PDPN tail can be phosphorylated62. To what extent these interactions regulate FRC function and LN microarchitecture remains unclear. The finding that CLEC-2 inhibited contractation to the same extent in Δcyto FRCs and wild-type FRCs suggested that CLEC-2 mediates its effect by preventing the extracellular domain of PDPN from interacting in cis with a lateral binding partner.

Similar to its action in FRCs, PDPN controls elongation and activation of RhoA in LECs45; however, LECs differed from FRCs in that LECs did not elongate upon binding CLEC-2. Furthermore, we found that bulk lymph flow and access of lymph to conduits were not altered upon in vivo blockade of PDPN. Given our results and the fact that LECs are not highly contractile, it is unlikely that PDPN expressed by LECs has a role in LN expansion and reticular architecture.

Cell contractility and sensing of matrix stiffness regulate the differentiation, migration and survival of fibroblasts44. The balance between the adhesion of a cell to a substrate of a given stiffness and the resulting tension within that cell determines overall tensional homeostasis44,45. Disruption of this balance, whether by changes in matrix stiffness or by changes in contractile potential, alters fibroblast proliferation46. Thus, the increased proliferation and survival that we observed for Pdpn–/– FRCs might have stemmed from dysregulated adhesion or tension or both.

Inflammation is known to trigger FRC proliferation; however, the timing and extent of division appears to vary between inflammatory stimuli, and the specific underlying mechanisms remain unknown34,47. Our data indicated that the relaxation of FRCs allowed early LN expansion in addition to providing a signal for proliferation, which caused FRCs to accumulate and accommodate the expanding organ. While a published study did not report changes in LN architecture57, we observed expansion of the FRC network upon immunization, as reported before34. This discrepancy could be due to differences in the timing or types of immunization used. It has been noted that PDPN amounts increase in response to select inflammatory stimuli57,48 (data not shown), which may restore high contractility to FRCs upon resolution of inflammation. Our results demonstrated that this process was crucial for FRC-mediated restraint of T cell proliferation.

Together, these data indicate that CLEC-2+ DCs provide a fast but reversible signal to PDPN+ FRCs. This allows transient relaxation of the FRC network that would persist only as long as CLEC-2+ DCs were crawling along the network. Ultimately, normal contraction of FRCs would resume shortly after removal of the CLEC-2 signal (and increased PDPN expression), which would occur as the immune response began to wane and migratory DCs persisted in the draining LN. Reestablishment of FRC contractility would enable structural and functional homeostasis in the reticular network to be restored.

**METHODS**

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

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**AUTHOR CONTRIBUTIONS**

J.L.A. and S.J.T. designed experiments, analyzed results and wrote the manuscript; J.L.A., V.C., J.F., M.C.D., J.R.P., J.M.N.-B., A.G., M.C.W. and R.M.W. performed experiments; J.F., K.S., Y.K., and L.X. supplied reagents and mice; L.O. and B.L. provided reagents and mice; and V.C., B.L., M.C.C., D.J.M. and L.X. provided comments on the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.
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ONLINE METHODS

Mice. C57BL/6 mice were purchased from Jackson Laboratory (strain 000664). Cd19-Cre mice were generated as described29 and were crossed to Rosa26-eYFP mice (strain 006148; Jackson Laboratory) to generate mice in which only FRCs express eYFP. The Cd19-Cre mice were also mated to B6;129-Gt(Rosa26Sor)1435b8J (B6)–/–, and Rosa26Sor (B6)–/– FRCs were plated in triplicate at a density of 2 × 10^5 cells per well. After 4–7 days of culture, the wells were washed three times with PBS and the absorbance of crystal violet was determined with a spectrophotometer at 570 nm.

FRC spreading and adhesion. For analysis of spreading, 10 cm dishes were coated with type I collagen according to the manufacturer's instructions (BD Biosciences). FRCs were plated and imaged with a light microscope at the appropriate times. Cell area was determined with ImageJ software. For analysis of adhesion, 96-well plates were coated with collagen, and 2 × 10^4 FRCs per well were added. At the appropriate times, the wells were washed three times with PBS for the removal of non-adherent cells, and the adherent cells were fixed in 4% PFA, then stained with crystal violet. After several washes for the removal of unbound crystal violet, the cells were lysed in 0.25% Triton-X, and the absorbance of crystal violet was determined with a spectrophotometer at 570 nm.

Immunoblot analysis. Purified FRCs were plated in 10 cm dishes at a density of 3 × 10^6 cells per plate and incubated overnight. Cells were starved of serum for 5 h and then were lysed in lysis buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycero; Thermo Scientific) containing protease and phosphatase inhibitor cocktails (Thermo Scientific). Protein concentrations were determined with the BCA protein assay kit (Thermo Scientific). Equal amounts of protein were loaded onto 4–12% Bis–Tris gels (Life Technologies) and separated by electrophoresis, then were transferred to a nitrocellulose membrane. Non-specific binding was blocked by incubation for 1 h in 5% nonfat milk, the membranes were incubated overnight with primary antibodies in 5% BSA. The following primary antibodies were used (all from Cell Signaling Technology): anti-ERK (1:1000), anti-phospho-ERK (1:1000), anti-AKT (1:1000), anti-phospho-AKT (1:1000), anti-RhoA (1:1000), anti-phospho-RhoA (1:1000), anti-cSource (1:1000), or anti-cSource (1:1000). The membranes were incubated for 2 h at 25 °C with horseradish peroxidase–conjugated rabbit–specific antibody (7074; Cell Signaling Technology). Finally, the signal was visualized with the SuperSignal WestDura Chemiluminescent Substrate (Fisher).

GTPase precipitation. For each condition, 7.5 × 10^5 wild-type, Pdpn−/−, or ΔcSource FRCs were plated in 10 cm dishes, and the activation of RhoA were determined with an Active RhoA Pull-Down and Detection kit according to the manufacturer's instructions (Thermo Scientific). Immunoblots were probed with anti-RhoA (Thermo Scientific) followed by horseradish peroxidase–conjugated anti-rabbit (secondary antibody) (identified above).

Gel contraction assay. For contraction assays, 1.0 × 10^5 cells were mixed with 50 μl Matrigel mix and were plated in 96-well culture dishes. After the gels had polymerized for 30 min at 37 °C, 100 μl medium was added to the wells, and gels were detached from wells of the plate with a sterile pipette tip. After 24 h, images of the wells were obtained and dimensions of gels were measured relative to well diameter, with ImageJ software. Alternatively, cells were allowed to seed the gel overnight, then CLEC-2–Fc or antibody 8.1.1 was added every 4–8 h and contraction was measured 24 h later.

Proliferation assays. Wild-type, Pdpn−/−, and ΔcSource FRCs were plated in triplicate at a density of 2 × 10^5 cells per well in a 96-well plate, and viable cells were quantified daily over the course of 4 d with the CellTiter-Glo Luminescent Cell Viability Assay according to the manufacturer's instructions (Promega). Data were normalized to the number of cells present on day 1 for each condition. For analysis of cell proliferation, 3.0 × 10^4 FRCs were labeled with 1 μM CFSE (carboxyfluorescein succinimidyl ester; Invitrogen) and were plated in six-well plates. On days 1, 2, 3, and 4, the cells were incubated with trypsin and then were analyzed by flow cytometry for quantification of the mean fluorescent intensity of the CFSE dye.

YAP staining. Wild-type or Pdpn−/− FRCs were plated on eight-well chamber slides and were allowed to adhere for 12 h to minimize the influence of differences in cell spreading on the quantification of nuclear YAP. Where indicated, CLEC-2–Fc was added to the medium for 15 min before seeding. After 12 h, the medium was changed, and fresh medium was added that either contained CLEC-2–Fc or did not. At each time point, cells were fixed for 15 min with 4% PFA and were washed three times for 5 min in PBS. Cells were immunostained with rabbit antibody to mouse YAP and TAZ (8418; Cell Signaling) and were visualized...
with Alexa Fluor 555–conjugated goat anti-rabbit (secondary antibody) (A-21428; Cell Signaling) according to the manufacturer’s instructions. Nuclei were counterstained with DAPI. The frequency of nuclear YAP was quantified with MATLAB software by summing of the fluorescence intensity within the region masked by the nucleus and the total fluorescence intensity of YAP staining on a per-cell basis.

In vivo blockade of PDPN. Mice were given intravenous injection (into the tail vein) of 100 μg purified anti-PDPN (8.1.1; Biologend) or the hamster IgG isotype-matched control antibody (SHG-1; Biologend). After 48 or 96 h, mice were killed and skin-draining LNs were collected for either flow cytometry or imaging. For CLEC–2-Fc experiments, 100 μg CLEC–2–Fc or rabbit IgG isotype-matched control antibody (identified above) was injected intravenously and LNs were collected 1 and 4 h later. Generally, flow cytometry was conducted on brachial LNs and imaging was conducted with axillary and brachial LNs or inguinal LNs for immunization experiments.

Compressive stress. Mice were treated with the hamster IgG isotype-matched control antibody or anti-PDPN (both identified above) 4 h before collection of LNs. Cylindrical discs (1.3 mm²) were punched from LNs with a biopsy punch to ensure a consistent cross-sectional area. These samples were subjected to compression with an Instron mechanical tester at a strain rate of 0.5 mm/min, and the stiffness was calculated as the slope of the first 10% of the resulting stress/strain curve.

Nuclei distance analysis. Rosa26Sor1Ythc xIlytFLyWMCd19-Cre mice were given an intravenous injection of 100 μg anti-PDPN or the isotype-matched control antibody (both identified above), and axillary and brachial LNs were collected 48 h later. LN sections were stained with antibody to green fluorescent protein (A-11122; Life Technologies) and Alexa Fluor 488–conjugated anti-rabbit (secondary antibody) (A-11034; Molecular Probes). Images of the FRC nuclei were collected and then were analyzed with CellProfiler software with the neighbor analysis module.

T cell–FRC interactions ex vivo. For quantification of T cells in contact with FRCs, Rosa26Sor1IlytCLyWMCd19-Cre mice were given an intravenous injection of anti-PDPN or an isotype-matched control antibody (both identified above). One day later, 5 × 10⁶ purified T cells labeled with Far Red cell tracker (Invitrogen) were injected intravenously. The mice were killed 24 h later, and LNs were fixed and imaged. Imaris software was used to generate isosurfaces for the T cells and the FRC network, and T cells in contact with the FRC network were quantified.

Immunization. For investigation of the role of PDPN during an immune response, BoyJ mice were first given an intravenous injection of anti-PDPN or an isotype-matched control antibody (both identified above). Two days later, 1 × 10⁶ CFSE-labeled CD45.2+ OT-1 T cells were injected intravenously into each mouse. Four hours later, mice received 500 μg of OVA and 30 μg of LPS intravenously, and a second dose of the isotype-matched control antibody or anti-PDPN (both identified above) was administered 24 h later. Mice were analyzed 24 and 48 h after administration of the OT-1 T cells, LPS, and OVA. Brachial LNs were collected and digested for flow cytometry analysis, while inguinal LNs were collected and fixed for imaging.

For immunization of Clec1b–/–Cd11c–Cre mice, PBS or 8 μg LPS was injected subcutaneously into the hind and forefootpads. LNs were collected 24 h later for flow cytometry (poptleal) or imaging (axillary and brachial).

Flow cytometry and antibodies. After the enzymatic digestion of LNs, cells were resuspended in flow cytometry buffer (PBS containing 2% FBS and 2 mM EDTA) and were incubated for 15 min with antibodies to the following surface markers: PDPN (8.1.1; Biologend), CD3 (MEC13.3; Biologend), CD45 (30–F11; Biologend), B220 (RA3–6B2; eBioscience), CD3e (17A2; Biologend), CD4 (RM 4-5; eBioscience) and CD8 (53-6-7; Biologend). For BrdU labeling, mice were given intraperitoneal injection of 2 mg BrdU 24 h before analysis. Cells were stained with a BrdU Flow Kit according to the manufacturer’s instructions (BD Pharmingen). Cells were then analyzed on a FACS caliber or FACS aria (both from BD Biosciences). Flow cytometry data were analyzed with FlowJo software.

Immunofluorescence staining of cells and tissue sections. Cells were plated on collagen-coated coverslips and were allowed to adhere overnight. Then, they were washed with PBS, fixed for 10 min in 4% PFA and permeabilized with 0.25% Triton-X before being stained with anti-PDPN (8.1.1; Biologend). LNs were collected, fixed for 4 h in 4% PFA and incubated overnight in 30% sucrose in PBS. LNs were frozen in optimal cutting temperature media (Fisher Scientific) on dry ice and then sections 20–80 μm in thickness were cut with a cryostat. Slides were stained immediately or were stored at −80 °C. Sections were fixed for 10 min in 4% PFA and permeabilized for 3 min with 0.25% Triton-X, and then nonspecific binding was blocked by incubation for 30 min with 2% BSA in PBS before incubation with antibodies. The sections were incubated for 1 h with anti-ER-TR7 (ab51824; AbCam), antibody to green fluorescent protein (identified above) and a biotin-conjugated antibody specific for peripheral node addressin (MECA-79; Biologend), then were washed three times with 2% BSA. The secondary antibodies Alexa Fluor 555–conjugated anti-rat (A-21434), Alexa Fluor 488–conjugated anti-rabbit (A-11034) and streptavidin–Alexa Fluor 647 (S-21374; all from Invitrogen) were then added for 30 min. After a final wash step, the slides were imaged on either a Leica SP5 X laser-scanning confocal microscope or a Zeiss 710 two-photon laser-scanning confocal microscope. Images were analyzed with ImageJ software. Surface area was analyzed with Imaris software (Bitplane). For each set of images acquired on the same day, the parameters for the surfaces module were set for the control samples and the same parameters were used for analysis of all images acquired in the same imaging session.

Tracer injection and conduit imaging. Wild-type mice were given an intravenous injection of isotype-matched control antibody or anti-PDPN (both identified above). After the injection, they were given a subcutaneous injection (into the footpad) of 10 μl saturated FITC solution (Sigma) in Hank’s balanced-salt solution (0.1 mg/ml). Popliteal LNs were collected 4 h after injection and fixed in 4% PFA. LNs were ‘optically cleared’ as described⁴⁹ and were mounted as whole-organ explants for multiphoton microscopy. Cortical and paracortical regions were identified by collagen density and the presence or absence of staining with CR1-specific antibody that recognizes follicular DCs (8C12; prepared in-house); 3 μg of this antibody was injected intravenously 6 h before the start of the experiment.

Isolation of platelets and BMDCs from fetal liver chimeric mice. Clec1b–/– fetal liver chimeras were generated as described³⁸. Fetuses from pregnant Clec1b–/– mice were collected from embryonic day 14.5 and embryonic day 18.5, and 1 × 10⁶ of their fetal liver cells were then injected retro-orbitally into lethally irradiated B6.SJL-PtprcPep3b/BoyJ mice (Jackson Laboratories). Mice were used 6–7 weeks later for experiments. BMDCs were generated as described³⁸. Bone marrow was collected from the tibias and femurs of the fetal liver chimeras and were cultured for 5–7 d in RPMI medium containing 10% FBS and 3% granulocyte-macrophage colony-stimulating factor. For studies in which BMDCs were injected, these cells were matured with 50 ng/ml LPS on day 7. Then, 1 × 10⁶ BMDCs were injected into the footpad and popliteal LNs were collected and weighed 1, 2 and 5 d later. Platelets were isolated as described³⁸. Approximately 500 μl whole blood was collected from the retro-orbital area of the fetal liver chimeras into 3.8% sodium citrate. Red blood cells were lysed in ACK lysis buffer (Fisher). Then, the cells were resuspended and centrifuged at 300g for 10 min with no brake. The supernatants were collected and centrifuged again at 1,000g for collection of the platelet-rich fraction. BMDCs and platelets were seeded into three-dimensional gels at a ratio of 5:1 to the FRCs.

Statistical analysis. Prism software (GraphPad) was used for statistical analyses. If data were not normally distributed or did not have equal variance between conditions, a Mann-Whitney U-test was performed. For normally distributed data, Student’s t-test was used. In some cases, a one-sample t-test was used to calculate whether the result for a sample was significantly different from 1. Data with a p-value of <0.05 were considered statistically significant. In cases in which multiple statistical tests were performed, a Bonferroni correction was used to determine a new α-cutoff for significance. Observers were not blinded to experimental conditions.

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