Mechanosensing by Peyer’s patch stroma regulates lymphocyte migration and mucosal antibody responses

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Fibroblastic reticular cells (FRCs) and their specialized collagen fibers termed ‘conduits’ form fundamental structural units supporting lymphoid tissues. In lymph nodes, conduits are known to transport interstitial fluid and small molecules from afferent lymphatics into the nodal parenchyma. However, the immunological contributions of conduit function have remained elusive. Here, we report that intestinal Peyer’s patches (PPs) contain a specialized conduit system that directs the flow of water absorbed across the intestinal epithelium. Notably, PP FRCs responded to conduit fluid flow via the mechanosensitive ion channel Piezo1. Disruption of fluid flow or genetic deficiency of Piezo1 on CCL19-expressing stroma led to profound structural alterations in perivascular FRCs and associated high endothelial venules. This in turn impaired lymphocyte entry into PPs and initiation of mucosal antibody responses. These results identify a critical role for conduit-mediated fluid flow in the maintenance of PP homeostasis and mucosal immunity.

RCs provide the architectural framework supporting lymphoid tissues. In lymph nodes (LNs), FRCs deposit and ensheath organized bundles of collagen fibers to form an intricate network extending from the subcapsular sinus to high endothelial venules (HEVs) and efferent lymphatics1,2. The anatomy of these structures allows for two functionally distinct compartments. The reticular network surface, comprised of interconnected FRC cell bodies, facilitates leukocyte attachment, cellular interactions and directional migration of leukocytes within the LN parenchyma, and is essential for lymphocyte homeostasis and the initiation of adaptive immune responses3–6. In contrast, the inner collagen core of the reticular network has been described as a conduit network, directing flow of lymph from the sinus into the LN parenchyma1. However, the functional and biological relevance of this latter aspect of the reticular network is less well established.

LN conduits have thus been proposed to function as an efficient and rapid pathway for transport of immunologically important soluble mediators from upstream tissues. Multiple reports have now demonstrated that lymph-borne antigen rapidly enters the LN conduit network, where it can be directly sampled by resident dendritic cells (DCs) and follicular B cells through gaps in the FRC sheath7–10. Likewise, soluble chemokines are rapidly delivered via the conduit network to the ablumenal surface of the HEV, whereupon these molecules are transcytosed and decorate the vascular lumen12–14. This process leads to an increase in leukocyte homing. This latter example has led to a predicted model in which conduits facilitate a form of ‘remote control’ wherein soluble mediators from upstream tissues can prime the LN for initiation of adaptive immune responses11.

The concept that FRC conduits mediate rapid and selective transport of soluble, small molecular weight material is widely accepted. However, the necessity for conduit-mediated molecular and fluid transport for either LN homeostasis or initiation of adaptive immune responses has not been addressed experimentally. So far, no models exist in which to interrogate lymph node function in the absence of conduits, nor have any physiological means of altering afferent lymph flow been developed, although surgical manipulation of upstream lymphatics has been reported15–17. Interestingly, LNs that have been surgically deprived of afferent lymph have been reported to exhibit a gradual ‘flattening’ of the HEV, loss of Glycam1 expression on the vascular endothelium and a corresponding decrease in lymphocyte immigration. However, the specific contribution of conduit-mediated lymph flow to this phenomenon cannot be ascertained, as surgical deprivation of afferent lymph additionally impacts cellular migration of antigen-experienced DCs from upstream tissues.

Here, we identify and describe FRC conduit networks in the intestinal PPs. Unlike lymph nodes, PPs lack a conventional source of afferent lymph that would normally contribute fluid flow through the conduit network. Instead, PPs are located at the interface of the intestinal lumen and directly absorb sample luminal contents, as opposed to lymph-borne materials. As with other regions of the intestine, the PP dome is lined with an absorptive epithelium that facilitates fluid transport through the establishment of local osmotic gradients. Here, we demonstrate that fluid absorbed in this manner enters a network of conduits that conduct the flow of fluid throughout the PP follicle and interfollicular regions. Additionally, we provide evidence that prolonged disruption of fluid flow through PP conduits, or loss of FRC responsiveness to conduit flow via the mechanosensitive ion channel Piezo1, led to profound alterations to homeostatic lymphocyte recruitment to the PP and a reduced capacity to mount mucosal antibody immune responses.

Results

PP conduits direct flow of absorbed luminal fluid. Despite lacking a conventional source of afferent lymph flow, characterization of...
stromal components in the small intestinal PP reveals that these lymphoid organs are supported by an intricate network of collagen-rich reticular fibers, structurally similar to the conduit network found in lymph nodes (Fig. 1). These fibers extend from the subepithelial dome and project into both the B cell follicle and interfollicular regions. Scanning electron micrographs of alkali-water macerated PPs reveal that these reticular fibers emerge directly from the disorganized meshwork of collagen overlying the PP dome, and eventually terminate directly along the blood vasculature (Fig. 1a). As with LN conduits, PP reticular fibers are ensheathed by a basement membrane marked by yellow arrows. Scale bar: 0.5 μm. d, Multiphoton microscopy of a PP from a Villin Cre-SSB-BFP mouse after oral gavage with soluble FITC and counterstained with anti-EpCAM1. IF, interfollicular region; F, follicle; Ep, epithelium. Scale bar: 50 μm. e, Multiphoton microscopy of a PP HEV. The mouse underwent oral gavage with soluble FITC and the HEV were stained by in vivo labeling with anti-MAdCAM before perfusion fixation. Data representative of images taken from at least two (a–c) or three (d,e) independent experiments.

A functional hallmark of LN FRC conduits is the ability to direct lymph flow from the lymph node sinus into the dense parenchyma of the cortex and paracortex, additionally facilitating delivery of soluble signaling molecules and antigen. The PP conduit network appears to perform a similar function, although drawing in fluid absorbed from the intestinal lumen in place of afferent lymph. Fluid uptake through PP conduits is identifiable through oral administration of soluble fluorescein isothiocyanate (FITC). Within 2h of gavage, FITC was detectable throughout the PP and selectively localized within the collagen-rich core of the conduit network (Fig. 1d).

High-magnification confocal imaging additionally confirmed that FITC-bearing conduits interface with blood vessels in the interfollicular region of the PP (Fig. 1e). Moreover, FITC signal is detectable along the blood vessel wall, suggesting a path of directional fluid flow from the intestinal lumen to the PP vasculature mediated by the PP conduit network. Together, these data demonstrate a functional reticular network of conduits supporting the intestinal PP. These conduits exhibit physical characteristics similar to those previously described in LNs, but uniquely function to facilitate fluid flow originating from the overlying intestinal epithelium as opposed to afferent lymph.

**Perturbation of intestinal fluid absorption disrupts PP conduit flow.** PP conduit flow appears to be uniquely dependent on fluid
absorption across the intestinal epithelium, a process that is tightly regulated by maintenance of local osmotic gradients through ion transport (Fig. 2a). The functional consequences of perturbed conduit fluid flow in the PP can thus theoretically be addressed by means of blocking fluid absorption. Here, disrupted fluid absorption was achieved by two mechanistically distinct treatments—first, by administration of a 10% solution of a high molecular weight polyethylene glycol (PEG) in drinking water, and second, by oral gavage with amiloride hydrochloride. PEG is a nonabsorbable, nonmetabolized, osmotically active substance that increases the osmolality of ingested fluid and promotes its retention in the intestinal lumen (Fig. 2b). By contrast, amiloride selectively disrupts the function of epithelial Na\(^+\)/H\(^+\) exchangers, thereby preventing the establishment of sufficient osmotic gradients for directional water transport (Fig. 2c). Both models of disrupted fluid absorption were found to restrict fluid uptake into PP conduits, as visualized by uptake of soluble FITC after gavage (Fig. 2d–f). Under these conditions, we were thus able to examine the state of PP structure and function in the absence of conduit-mediated fluid flow.

**Impaired fluid absorption disrupts HEV structure.** Extended treatment of mice with PEG in drinking water for a period of up to 3 d does not result in gross changes to the overall architecture or total numbers of FRCs (Supplementary Fig. 1a), suggesting that fluid flow is not necessary for the maintenance of the FRC network itself. However, treatment with PEG for periods beyond 1 week resulted in a specific decrease in blood endothelial cell (BEC) numbers, including endothelial cells (ECs) of the PP HEV, identified by expression of mucosal addressin cell adhesion molecule 1 (MAdCAM1) (Supplementary Fig. 1b).

Changes to HEV EC cell numbers is preceded by a striking alteration in HEV structure. An intact PP HEV is structurally comprised of MAdCAM-expressing endothelium closely encircled by a ring of perivascular FRCs (Fig. 3a,c). Confocal imaging of the PP HEV following a 3-d treatment period with PEG revealed an apparent disruption in the normally continuous perivascular FRC ring, and a quantifiable decrease in contact points between perivascular FRCs and HEV ECs (Fig. 3a). These structural alterations are also apparent by high-magnification transmission electron microscopy (TEM) of the PP HEV, which show distinct points of separation between the HEV ECs and the surrounding basement membrane (Fig. 3b). Interestingly, these TEM images additionally revealed an apparent dearth of leukocyte interactions with the vascular endothelium, as few cells were found either attached to the HEV lumen or within HEV pockets. This latter observation suggests a potential functional deficiency in HEV-mediated leukocyte immigration corresponding to perturbations in HEV structural integrity. In total, these data suggest that the absence of directional conduit flow disrupts the alignment of perivascular FRCs along the HEV (Fig. 3c).

**Impaired fluid absorption disrupts lymphocyte recruitment.** HEVs are the main port of entry for naïve recirculating lymphocytes...
Additionally, alteration to PP cellularity was limited to recirculating and T lymphocytes were affected equally (Supplementary Fig. 2a). Increases to LN cellularity may be a consequence of dehydration. Rather, the process of fluid uptake via the oral route appears to be necessary for maintaining normal patterns of lymphocyte migration and homeostasis.

Impaired lymphocyte rolling on the HEV. Lymphocyte ingress across HEVs to lymphoid tissues is a multistep process, which involves, first, selectin-mediated capture and rolling along the vascular endothelium, followed by integrin- and chemokine-mediated firm arrest and transmigration through endothelial cell junctions. Intravital multiphoton imaging of PPs following adoptive transfer of labeled lymphocytes revealed a near-complete absence of lymphocyte rolling events on the HEV lumen of mice treated with PEG or amiloride (Supplementary Videos 1 and 2). By contrast, several lymphocyte rolling and adhesion events were identifiable within the HEVs of control mice (Supplementary Video 3).

Lymphocyte rolling in intestinal PPs is largely mediated through interaction between MAdCAM1 expressed by HEV ECs, and either l-selectin or the integrin α4β7 on recirculating lymphocytes. Similar to treatments with PEG or amiloride, intravenous administration of a MAdCAM1-blocking antibody eliminated lymphocyte rolling events on the HEV (Supplementary Video 4). Moreover, anti-MAdCAM1 treatment limited short-term lymphocyte accumulation in the PPs, but not in LNs (Fig. 5a), and anti-MAdCAM1 blockade over the course of 3 d resulted in an overall decrease in PP lymphocyte cellularity that was comparable to that seen following PEG and amiloride treatment (Fig. 5b). Combining PEG and anti-MAdCAM treatments did not result in an additive decrease to either short-term accumulation of circulating lymphocytes or overall cellularity in the PP (Fig. 5a,b). As such, we consider the possibility that these treatments are mechanistically redundant.
Despite noticeable changes to HEV structure following a 3-d treatment with PEG, RNA-seq analysis of flow cytometry-sorted HEV ECs and FRCs at this time point revealed few significant alterations in the transcriptional profile. Notably, HEV EC expression of genes encoding key adhesion molecules involved in lymphocyte capture and recruitment, including *Madcam1*, was not significantly altered (Fig. 5c). Likewise, PP FRCs, which direct lymphocyte capture and recruitment, including *Madcam1* of genes encoding key adhesion molecules involved in lymphocyte migrations in the transcriptional profile. Notably, HEV EC expression as the total number of adoptively transferred cells colocalizing with MAdCAM1 as the total number of adoptively transferred cells colocalizing with MAdCAM1 (left) or the percentage of adoptively transferred cells colocalizing with MAdCAM1+ HEVs (right) (n = 23 (ctl) or n = 17 (PEG) PP, isolated from five mice per group). *c*. Flow cytometric analysis of relative lymphocyte cellularity in the PP, MLN, ILN or spleen of untreated control (ctl), PEG-treated (PEG) or amiloride-treated (aml) mice (N = 9 (ctl), 12 (PEG, aml) mice). For all graphs, data represented as mean ± s.e.m. from three (a,c,d) or two (b) independent experiments. Groups were compared by one-way ANOVA (a,d) or unpaired two-tailed Student’s *t*-test (c). *P* < 0.05, **P** < 0.01, ***P*** < 0.0001. NS, not significant.

**Fig. 4 | FRC responsiveness to PP conduit fluid flow is necessary to maintain lymphocyte recruitment and PP homeostasis.** *a*. Flow cytometric analysis of adoptively transferred splenocytes accumulating in the PP, MLN, ILN and spleen 1 h after transfer into control (ctl), PEG-treated (PEG) or amiloride-treated (aml) recipients (N = 8 (ctl) or 7 (PEG, aml) mice per group). *b*. Flow cytometric analysis of adoptively transferred splenocytes accumulating in the PP and LN of an untreated recipient mouse. Adoptively transferred splenocytes were obtained from control and PEG-treated donors and transferred in a 1:1 ratio (n = 5 mice per group). *c*. Confocal microscopy of PP from control or PEG-treated recipient mice 1 h after adoptive transfer of CellTracker Green-labeled splenocytes. Representative images, costained with anti-PDPN (gray) and anti-MAdCAM1 (red). Scale bar: 100 μm. Quantification of imaging represented as the total number of adoptively transferred cells colocalizing with MAdCAM1+ HEVs and the percentage of adoptively transferred cells colocalizing with MAdCAM1+ HEVs (right) (n = 23 (ctl) or n = 17 (PEG) PP, isolated from five mice per group). *d*. Flow cytometric analysis of relative lymphocyte cellularity in the PP, MLN, ILN or spleen of untreated control (ctl), PEG-treated (PEG) or amiloride-treated (aml) mice (N = 9 (ctl), 12 (PEG, aml) mice). For all graphs, data represented as mean ± s.e.m. from three (a,c,d) or two (b) independent experiments. Groups were compared by one-way ANOVA (a,d) or unpaired two-tailed Student’s *t*-test (c). *P* < 0.05, **P** < 0.01, ***P*** < 0.0001. NS, not significant.

While transcriptional expression of *Madcam1* appears to be unaffected by altered PP conduit flow, there was a striking difference in the amount of MAdCAM1 protein exposed on the luminal surface of HEVs. Lumenally exposed MAdCAM was specifically visualized by a short pulse of anti-MAdCAM1 (MAdCAM 488) delivered intravenously to control and PEG-treated animals. Substantially less in vivo labeling with anti-MAdCAM1 was observed in PEG-treated animals relative to control (Fig. 5d). Quantification of the percentage of lumenally exposed MAdCAM1 was performed by comparing in vivo labeled anti-MAdCAM1 (MAdCAM-488) signal to total anti-MAdCAM1 (MAdCAM-PE, visualized by post-fix staining; PE, phycoerythrin). Under normal conditions, nearly all MAdCAM1 was exposed on the luminal surface of HEVs. By contrast, this frequency drops to roughly 60% following PEG treatment (Fig. 5e). These data suggest that localization of MAdCAM1 protein, but not transcriptional expression, is altered in the absence of conduit fluid flow.

**Blockade of fluid absorption impairs mucosal antibody responses.** Access of lymph-borne material to the conduit network of lymph nodes is restricted to soluble molecules below a molecular weight cutoff of roughly 70 kDa (ref. 13). Interestingly, this cutoff does not apply to molecules locally produced or directly injected...
into the lumen itself, indicating that the physical properties of conduits themselves are not limiting. Rather, size selectivity in LN conduits is a property conferred by sinus-lining endothelial cells, which contain transendothelial channels physically gated by fibrils formed by the glycoprotein PLVAP.

In contrast to LN conduits, the conduit network of PPs is not covered by these sinus-lining endothelial cells, but rather a monolayer of absorptive epithelium and microfold cells (M cells). The extent to which soluble antigen may access the PP conduit network, whether this access is size selective and the impact of this mode of antigen transport on mucosal immune responses has not previously been examined. To address this question, we co-administered a small 14-kDa hen egg lysozyme (HEL) antigen labeled with Alexa Fluor 488 (HEL-488) and a large 250-kDa HEL labeled with phycoerythrin (HEL-PE) to the lumen of explanted intestinal loops. Confocal imaging of the PPs revealed that the 14-kDa HEL (HEL-488) rapidly penetrates the PP dome and tracks along the FRC conduit network, while HEL-PE primarily remained near the epithelial surface and appeared instead to be carried into the PP follicle by myeloid cells (Fig. 6a). These data suggest that, as with LNs, conduit-mediated antigen transport within intestinal PPs appears to be limited to small molecular weight molecules.

To examine the immunological role of conduit-mediated anti- gen transport in PPs, PEG-treated and control mice were orally immunized with two nitrophenyl (NP)-haptenated protein antigens of differing molecular weights: 14 kDa NP-conjugated HEL (NP-HEL) and 150 kDa NP-conjugated chicken gamma globulin (NP-CGG). Given the size exclusivity of molecular transport through the PP conduit network, PEG-mediated blockade of fluid absorption and conduit flow should alter the route of intrafollicular transport for NP-HEL, while transport of the much larger NP-CGG should be absent from PP conduits in both PEG-treated and control immunized mice. Following immunization, anti-NP fecal IgA titers were reduced in mice that had been concurrently treated with PEG to impair PP conduit flow (Fig. 6b). Interestingly,
however, antibody responses to NP-HEL and NP-CGG were equally affected by treatment with PEG. Thus, while limitations in conduit flow can impact antigen-specific humoral responses in the PP, this is not necessarily a consequence of alterations in the mode of antigen transport.

Total fecal IgA titers were likewise found to be lower in mice treated with PEG over the course of 2 weeks relative to control mice (Fig. 6c), and these mice exhibited a decrease in baseline germinal center (GC) cell activity (Fig. 6d). Under normal conditions, PPs exhibit continuous ongoing GC reactions regulated by an extensive network of follicular dendritic cells (FDCs). FRC interactions with FDCs have previously been noted in the context of antigen transport by LN conduits, and as PP conduits extend into the B cell zone of the PP we considered the possibility that disrupted conduit flow may impact the FDC network or GC activity\(^{22}\). While GC activity was significantly decreased, the PP FDC network appeared structurally normal (Fig. 6e). Together, these data suggest a more general decrease in immune responsiveness to mucosal antigen when conduit flow is disrupted.

We speculate that this reduction may be a direct consequence of impaired homeostatic recruitment of naïve lymphocytes to the PP following loss of conduit flow to the HEV. As previously established, anti-MAdCAM treatment directly impacts lymphocyte recruitment to the PP in a manner similar to PEG or amiloride treatment, but does so without any additional effects that might be associated with perturbed fluid absorption. We thus tested antigen-specific antibody responses in mice treated with anti-MAdCAM1 blockade during oral immunization. Under these conditions, mice exhibited fewer NP-specific B cells in the intestinal lamina propria (Fig. 6f,g), as well as a reduction in anti-NP IgA titers relative to control animals (Fig. 6h). Moreover, this reduction in antibody response was similar to that seen in mice immunized during PEG treatment. In total, these data suggest that the amplitude of PP-derived mucosal immune responses is affected by the state of PP lymphocyte cellularity.
Piezo1 confers responsiveness to conduit flow in PP FRCs. Mechanical cues associated with directional fluid flow may be necessary for proper orientation of the HEV and surrounding perivascular networks of FRCs. Previous reports have demonstrated that vascular integrity may depend on expression of the mechanosensitive ion channel Piezo1 by endothelial cells, which confers morphological responsiveness to the sheer force exerted by vascular flow [22, 23]. While PP conduits terminate at the abluminal surface of HEVs, disrupted conduit-mediated fluid flow is unlikely to appreciably alter the rate of vascular flow or EC responsiveness. However, we find that FRCs likewise express Piezo1 transcript, although at lower abundance than HEV ECs, and thus we examined the possibility that FRCs may be similarly responsive to conduit-mediated fluid flow via Piezo1 signaling (Supplementary Fig. 4a).

Conditional Piezo1-deficient mice were generated by crossing mice expressing Cre recombinase directed by the Ccl19 promoter...
Ccl19-PEG-treated and Ccl19−/− mice (Supplementary Fig. 6b). Importantly, specificity of Cre recombinase activity was tracked by alternatively crossing Ccl19cre mice with a ROSA26-eYFP reporter mouse, and found to be largely restricted to PDPN-expressing stromal cells, with almost no expression in either lymphatic endothelial cells (LECs) or BECs (Supplementary Fig. 4c). In contrast to the LN, a significantly smaller proportion of PDPN−/CD31− cells isolated from PPs exhibit Ccl19 reporter activity. However, these cells readily expand in culture and exhibit typical FRC morphology, and transcriptional expression of Piezo1 was significantly ablated in culture-expanded FRCs isolated from Ccl19−/− mice (Supplementary Fig. 4d,e).

Confocal imaging of PPs from these mice exhibited eYFP signal localized to PDPN+ FRCs within the PP follicle and interfollicular region (Supplementary Fig. 5a). Additionally, we found clear reporter expression in FRCs surrounding the HEV, but no expression in HEV ECs (Supplementary Fig. 5b). By contrast, minimal eYFP signal was found within RANKL+ marginal reticular cells (MRCs) located in the subepithelial dome. Previous reports have suggested that FDCs may express low levels of Ccl19 and have demonstrated variable levels of Ccl19 activity within the PP FDC network24,25. Here, we find that, while the majority of the FDC network, identified as complement receptor-positive (CR1/CR2+), was negative for reporter expression, a small subset of FDCs near the FRC–FRC interface may in fact be positive for eYFP expression, and thus we cannot rule out the possibility that FDCs may also be partially targeted in these mice.

LNs and PPs develop normally in these mice, and the stromal cell compartment was largely unchanged relative to Ccl19cre Piezo1lox/lox (wild type, WT) controls (Supplementary Fig. 6a). However, as with PEG-treated animals, we found that conditional ablation of Piezo1 resulted in visible structural aberrations of the HEV-associated perivascular FRCs (Fig. 7a). Additionally, expression of MAdCAM1 exposed on the luminal surface of the HEV endothelium was found to be significantly reduced in Ccl19−/− mice relative to controls, suggesting a potential link between HEV polarization and Piezo1-mediated sensation of conduit fluid flow in HEV-supporting FRCs (Fig. 7b). In contrast to the HEV, LYVE1+ efferent lymphatics, along with the FRCs that interface with these vessels, appeared structurally normal in both PEG-treated and Ccl19−/− mice (Supplementary Fig. 6b).

As with PEG-treated mice, alterations to HEV structure in Ccl19−/− mice were associated with reductions in lymphocyte recruitment to the PPs in Ccl19−/− mice, as well as a cumulative decrease in overall lymphocyte cellularity (Fig. 7c,d). This perturbation in lymphocyte recruitment affected both naïve circulating B cell and T cell populations (Supplementary Fig. 6c). Interestingly, Ccl19−/− mice also exhibited increases in lymph node cellularity in a manner similar to that observed following PEG or amiloride treatment, despite LN FRCs expressing similar baseline levels of Piezo1 transcript to PP FRCs (Fig. 7c,d and Supplementary Fig. 6d). This suggests that LN FRCs may not rely on Piezo1 to support homeostatic maintenance or accumulation of lymphocytes to LNs. Finally, oral immunization of Ccl19−/− mice against NP-CGG resulted in less potent mucosal IgA responses as compared to wild-type controls. Fewer NP-specific B cells were present in the intestinal lamina propria (Fig. 7e,f), and NP-specific fecal IgA titers were significantly lower (Fig. 7g).

Cumulatively, these data suggest that mechanosensation of conduit-mediated fluid flow via Piezo1 contributes to the proper alignment of PP FRCs, particularly at the site of FRC–HEV interaction where conduits terminate along the vessel’s abluminal surface. Loss of Piezo1 signaling within Ccl19-expressing stroma of PPs results in structural aberrations at HEVs, impaired recruitment and accumulation of lymphocytes to PPs, and a reduced capacity to establish mucosal antibody response against antigen.

**Discussion**

Various descriptions of the reticular network functioning as a conduit for the transport or distribution of soluble material have been reported over the last several decades17–20, leading to speculation of immunologically important roles for conduit-mediated transport of chemokines and antigen18–20. However, exploration of LN conduit networks thus far has been largely restricted to interpretation of in situ imaging, and thus our understanding of conduit networks remains purely descriptive. It has proven far more challenging to examine experimentally the biological importance of conduits and conduit-mediated fluid transport.

So far, the only means of effectively depriving a LN of conduit flow relies on the surgical ligation of afferent lymph vessels—a process that deprives LNs not only of lymph flow and lymph-borne soluble mediators, but also of tissue-derived migrating DCS as well17–20. Thus, the specific contributions of disrupted conduit flow cannot be definitively established by this experimental approach. Here, we sought to gather insight into the biological importance of fluid flow through a reticular conduit network sustaining intestinal PPs, which is uniquely linked with the process of epithelial fluid absorption and thus more experimentally pliable than that of LN conduits. Following prolonged disruption of intestinal fluid absorption, we found that the PP HEV structure is perturbed and lymphocyte recruitment and cellularity decrease substantially. While disruption of fluid absorption negatively impacts lymphocyte recruitment to PPs, we also noted a surprising increase in cellularity of LNs. This increased LN cellularity may result from a redistribution of circulating lymphocytes, which might otherwise accumulate in the PP.

Interestingly, these HEVs also exhibited a decrease in the display of MAdCAM1 at the vascular lumen, but few alterations to transcriptional profile, suggesting an alteration to endothelial polarization rather than impaired HEV differentiation. FRCs, which line the conduit network and interface with the HEV, likewise exhibit few transcriptional alterations. However, evidence that both FRCs and HEVs may be functionally and morphologically affected by the mechanical force exerted by fluid flow have been previously reported12,21,22. Mechanosensation of vascular fluid flow by ECs, for instance, has been attributed to expression of Piezo1. Here, we examined the impact of Piezo1 signaling specifically in Ccl19-expressing stromal cells, which consist primarily of FRCs and a small subset of FDCs, to determine whether these cells similarly sense and respond to conduit-mediated directional fluid flow. Indeed, we found many alterations to PP homeostasis that closely resemble the effects of PEG- or amiloride-induced perturbations in fluid absorption and thus suggest that expression of Piezo1 by PP FRCs confers responsiveness to conduit-mediated fluid flow in the PP.

These alterations to lymphocyte trafficking demonstrate the functional importance of conduit networks for the homeostatic maintenance of lymphoid tissues. However, we have additionally demonstrated that blockade of fluid absorption and directional conduit flow negatively impacts the amplitude of antigen-specific mucosal IgA responses. A role for conduit-mediated transport of IgM antibodies secreted locally in the lymph node has recently been demonstrated, suggesting that conduit transport may be a key contributor to antibody responses18. Conduits in the PP may likewise contribute to the export of locally produced antibody. However, we additionally observed a decrease in PP GC activity, as well as decreases in the number of antigen-specific B cells in mice orally immunized against NP-haptenated protein. These data suggest that loss of conduit flow impacts the initiation of B cell responses as well.

The mechanistic link between PP conduit flow and initiation of B cell responses is not entirely clear. It has also been proposed that conduits may contribute to immune responses, by providing an alternative route for the transport of antigen. However, we found no evidence
that the size of antigen or its transport through PP conduits impacts the resulting immunological response. It is additionally possible that the alteration of the FDC network, which may intersect with the conduit network, may be a contributing factor to altered mucosal responses following impaired conduit flow\cite{11}. While we have not observed any clear alterations to the size or structure of the FDC network, the function of FDCs in this context may warrant further investigation.

An alternative explanation for decreases in IgA responses following blockade of fluid absorption is that impaired lymphocyte recruitment to the PP restricts the pool of naive lymphocytes locally available to respond to antigen. Previous studies quantifying the primary immune B cell and T cell precursor populations have demonstrated a striking correlation between the size of an antigen-reactive precursor population and the amplitude of primary immune responses to cognate antigen on immunization—the larger the naive precursor population, the more robust the primary adaptive immune response\cite{25, 26}. By extension, the total number of antigen-reactive naïve B cells available in the PP at the time of immunization may directly influence the amplitude of resultant immune responses. In line with this concept, the effects of impaired fluid absorption on mucosal antibody responses can be largely recapitulated in mice treated systemically with anti-MAdCAM or in Ccl19-P1cKO mice, wherein lymphocyte cellularity of the PP is reduced, but fluid absorption and conduit flow are normal. The clear shift in lymphocyte population distribution away from the PPs and towards LNs might therefore broadly impact the nature of the immune responses an individual may be prepared to elicit.

Cumulatively, we have shown that the PP conduit network plays an integral role in supporting PP immune homeostasis. The loss of conduit flow not only impacted lymphocyte recruitment and PP cellularity, but also culminated in decreased capacity to mount mucosal immune responses. Due to the unique link between PP conduit flow and intestinal fluid absorption, we anticipate that these processes may be particularly relevant when considering conditions characterized by fluid malabsorption, such as inflammatory or infectious diarrheal diseases and chronic laxative use. Ultimately, the contribution of fluid absorption and PP conduit fluid flow within the greater context of mucosal health and disease should be a topic of great interest for future investigation.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41590-019-0505-z.

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**Author contributions**

J.E.C. designed and performed experiments, analyzed results and wrote the manuscript. M.B.B. performed experiments, analyzed results and contributed to the writing of the manuscript. E.G. performed experiments and analyzed results. M.C.C. and S.J.T. designed and supervised the study and contributed to the writing of the manuscript.

**Competing interests**

S.J.T. and M.B.B are employed by Genentech.

**Additional information**

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Supplementary information is available for this paper at https://doi.org/10.1038/s41590-019-0505-z.

Correspondence and requests for materials should be addressed to S.J.T. or M.C.C.

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Methods

Mice. Experiments were performed in C57BL/6 mice unless otherwise indicated. C57BL/6 mice, VillinCre mice (021504) and Piegofl/fl mice" (029213) were purchased from Jackson Laboratories. Rosa26-SSB-BFP mice were generated in the laboratory and previously described. VillinCre mice were crossed to Rosa26-SSB-BFP line to generate VillinCre-SSB-BFP mice. Cd19Cre mice were obtained from B. Ludewig (Kantonsspital St. Gallen) and have been previously described. Cd19-PtcKO mice were generated by crossing Cd19cre−/− heterozygous mice with Piegofl/fl homozygous mice twice to generate Cd19cre−/− Piegofl/fl breeders. These mice were then crossed with Piegofl/fl mice to generate either Cd19cre−/− Piegofl/fl (Cd19-PtcKO) or Cd19cre−/− Piegofl/fl (WT) offspring for experimentation. All resulting offspring are of mixed C57BL/6N and C57BL/6J background. Cre-negative littersmates were used as wild-type controls in each experiment. Mice were maintained under specific pathogen-free conditions in accordance with institutional and National Institute of Health guidelines and used at 6–8 weeks of age. For each of the experiments performed, age-matched mice of both sexes were analyzed. Experiments were approved by the Boston Children’s Hospital and Harvard Medical School institutional animal use and care committee in accordance with National Institute of Health guidelines for the humane treatment of animals.

Antibodies. The following antibodies were used: anti-CD45 (30-F11), anti-CD31 (390), anti-PPDPN (8.1.1), anti-Madcam-1 (MECA-367), anti-B2a2 (R200-3A6-B2), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD8α (53-6.7), anti-EpCam (G8.8), anti-CD3ε (145-2C11), anti-TER119 (TER119), anti-Cri/Cri (7E9), anti-GL7 (G17) and anti-CD58 (90) from BioLegend, anti-perlcan (A7/6) from Thermo Fisher Scientific, anti-IGα (C10-3) from BD Biosciences and anti-Col1 (Millipore Sigma).

Disruption of fluid absorption. Fluid absorption was disrupted by one of two treatments: 1) PEG was administered to mice ad libitum through drinking at a concentration of 10% (v/v) for a duration of up to 2 weeks, depending on the experiment, or 2) mice were orally gavaged with amiloride (0.1 mg kg−1) once daily for up to 3 days, depending on the experiment.

Immunohistochemistry and confocal microscopy. Isolated tissues were fixed in 4% paraformaldehyde (PFA) for 4 h and placed in 30% sucrose in paraformaldehyde solution for cryoprotection and then frozen in OCT (optimal cutting temperature) medium. Tissue was embedded in OCT (optimal cutting temperature) medium, frozen and cut into 20-μm sections. Sections were stained and imaged using a Zeiss Axioskop 2 Plus microscope, and images were analyzed using ImageJ. Immunohistochemistry was performed with cell adoptive transfer. For experiments examining long-term effects on PP tissue, mice were euthanized 10 min after retro-orbital injection of 0.1 mg kg−1 potassium ferrocyanide (K4Fe(CN)6) for 1 h, washed in water three times and incubated in 1% aqueous uranyl acetate for 1 h, followed by two washes in water and subsequent dehydration in alcohol series of 70%, 90%, 100%, 2×100% and 3×100%. Ultrathin sections (70–90 nm) were cut on a Leica EM UC6 ultramicrotome. Sections were washed in water three times and incubated in 1% osmium tetroxide (OsO4) for 1 h, washed in 0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide (OsO4)/1.5% potassium ferrocyanide (K4Fe(CN)6) for 1 h, washed in water three times and incubated in 1% aqueous uranyl acetate for 1 h. Samples were then placed in propyleneoxide for 1 h and subsequently infiltrated overnight in a 1:1 mixture of propyleneoxide and TAAB Epon (Marivac Canada). The following day the samples were embedded in TAAB Epon and polymerized at 60°C for 48 h. Ultrathin sections (about 60 nm) were cut on a Reichert Ultracut-S microtome, placed onto copper grids, stained with uranyl acetate and lead citrate and examined on a JEOL 1200EX electron microscope. Images were recorded with an AMT 2k CCD camera.

Conduit analysis. Mice were gavage fed with 400 μl of FITC-saturated PBS solution 2–4 h before collecting intestine. To examine the effects of blockade of fluid absorption, mice were: (1) gavage fed with 400 μl of FITC-saturated PBS solution with 10% PEG, or (2) gavage fed with FITC in PBS 2 h after gavage treatment with amiloride (0.1 mg kg−1). The intestinal lumen was washed by lavage with 10 ml of ice-cold PBS. PBS was then excised and fixed in 4% PFA. Fixed tissues were immersed in 30% sucrose for cryoprotection and then frozen in OCT medium. Thick, 100-μm sections were cut using a cryostat. Sections were imaged by multiphoton microscopy.

Adoptive transfer of lymphocytes. A single-cell suspension of naïve lymphocytes was prepared from spleens and C57BL/6 donor mice and immediately labeled with CellTracker Green CMFDA dye (Thermo Fisher Scientific, catalog no. C2925). Lymphocytes were adoptively transferred to either untreated control, PEG-treated or amiloride-treated recipient mice by retro-orbital injection in 50 μl of sterile saline. Alternatively, lymphocytes were adoptively transferred in sterile saline containing 20 μg of anti-Madcam1. For comparison of lymphocytes from untreated control and PEG-treated-donor mice, donor spleen cells were similarly collected and labeled, and then mixed in a 1:1 ratio before adoptive transfer to recipient mice. For all experiments, mice were euthanized 1 h after adoptive transfer, and PPs and ILNs were collected for analysis.

FTY720 treatments. To prevent cell egress, mice were injected with 1 mg kg−1 FTY720 (Fingolimod, R&D Systems) intraperitoneally immediately before the start of treatment with PEG or amiloride. Mice received an additional dose of FTY720 at 48 h and were killed for analysis at 72 h.

Antibody blockade of MadCAM1. To block lymphocyte interactions with the PEG PEV, mice were injected intravenously with 20 μg of anti-MadCAM1 (MECA-367). For experiments examining short-term recruitment of adoptively transferred lymphocytes, mice were given a single injection simultaneously with cell adoptive transfer. For experiments examining long-term effects on PP cellularity and antibody response, mice were injected once daily with 20 μg.

In vivo labeling of MadCAM1. Control or PEG-treated mice received 20 μg of anti-MadCAM1 (488) by retro-orbital injection. Mice were euthanized 10 min after injection and perfused with ice-cold 4% PFA. PPs were collected and drop-fixed in
PFA for an additional 4 h, immersed in 30% sucrose overnight and frozen in OCT medium. Tissue sections were then stained with anti-MAdCAM1-PE and analyzed by confocal microscopy. Images were imported to FlowJo software, and pixel intensity for either 488 or PE channels was assessed for each pixel. Data were quantified as percentage of pixels positive in the 488 channel out of all stained pixels (488 + PE).

**Image quantification of HEV-associated lymphocytes.** Confocal imaging was performed on PPs collected from mice following adaptive transfer of CMFDA-labeled lymphocytes. Sections were counterstained with anti-MAdCAM to label HEVs. Masks of HEV staining were created in ImageJ software and lymphocyte numbers within masks were quantified as lymphocytes associated with the HEV.

**Image quantification of HEV–FRC contact points.** Confocal imaging was performed on PPs collected from either B6 mice following treatment with PEG, or from Ccl19-PickKO mice. Sections were stained with anti-MAdCAM to label HEVs and anti-PDPN to label FRCs. Image analysis was performed in ImageJ. Binary images of each channel (HEV and FRC) were generated by thresholding. A perimeter of the HEV was generated by dilating the HEV threshold image once, and then subtracting the original HEV threshold. Contact points were determined as all pixels in which the FRC threshold image overlapped with the HEV perimeter. Data are represented as: (contact points)/(HEV perimeter).

**Oral immunization and mucosal antibody responses.** For analysis of antigen-specific mucosal antibody responses, control (untreated) or PEG-treated animals received either NP-HEL (100 μg) or NP-CGG (20 μg) once daily by oral gavage for 2 weeks. Unimmunized controls received PBS once daily by oral gavage for the same duration. All mice were allowed to recover on normal drinking water for 3 d following the final immunization. Stool was collected for fecal lgA analysis. Intestinal tissue was collected for enzymatic digestion and analysis by flow cytometry. Fecal lgA was extracted as follows. Fecal pellets were homogenized with a Tissulyser LT bead mill (Qiagen) in a solution of protease inhibitor cocktail in PBS for 10 min. Nonsoluble fecal material was removed by two rounds of centrifugation at 4 °C. Supernatant containing soluble fecal lgA was reserved for analysis by ELISA, while the pellet was allowed to dry and subsequently weighed. Sample volume was normalized to fecal dry weight. ELISA analysis was performed through immobilization of NP-BSA on a high-binding plate (Greiner Bio-One, 655081), addition of fecal lgA preparation at twofold serial dilutions and performed through immobilization of NP-BSA on a high-binding plate (Greiner Bio-One, 655081), addition of fecal lgA preparation at twofold serial dilutions and probing for specific binding of goat polyclonal anti-lgA-AP (Southern Biotech) using standard alkaline phosphatase development. For analysis of total fecal lgA, high-binding plates were coated with goat polyclonal anti-lgA capture antibody (Southern Biotech), followed by addition of fecal lgA preparation and probing for specific binding of rat anti-IgA-AP (11-44-2) by alkaline phosphatase development. Specific binding of rat anti-IgA-AP (11-44-2) by alkaline phosphatase development.

**Analysis of NP-specific B cells.** Small intestines were collected from mice following oral immunization against NP-CGG. PPs were removed and fat was carefully dissected away. The intestine was cut into 2–3 cm long segments and inverted using forceps. Intestinal segments were then washed twice in 2 mM EDTA and 5% dithiothreitol in PBS at 37 °C for 20 min each to remove epithelium. The remaining lamina propria was enzymatically digested at 37 °C in RPMI containing 0.1 mg/ml−1 DNase I (Invitrogen), 0.2 mg/ml−1 collagenase P (Roche) and 0.8 mg/ml−1 dispase (Roche) for 50–60 min. NP-specific B cells were then identified by incubation with NP-PE for 20 min on ice, then analyzed by flow cytometry.

**Statistical analysis.** All averaged data are presented as ± s.e.m. Statistical tests were performed using GraphPad Prism software. For experiments comparing two treatment groups, a two-tailed, unpaired Student’s t-test was performed. For comparison of three or more treatment groups, a one-way analysis of variance (ANOVA) was performed followed by Tukey’s post-hoc multiple comparison tests. Differences were considered to be statistically significant when P < 0.05. For graphs, data are shown as mean ± s.e.m. Statistical significance is indicated by the following: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.005. No statistical significance is indicated with ‘NS.’

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The data that support the findings of this study are available from the corresponding author upon request. Microarrays are available on the Gene Expression Omnibus database with the accession number GSE135612.

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Software and code

Policy information about availability of computer code

Data collection

Flow Cytometry = BD FACS DIVA software; Confocal and Multi photon imaging= Olympus Fluoview

Data analysis

Flow Cytometry Analysis in FlowJo (Flowjo, V10.3)
Image Processing in ImageJ
Data Analysis in Microsoft Office Excel 2007
Statistical Analysis in GraphPad Prism v6

RNAseq libraries were sequenced on an Illumina HiSeq. RNA-sequencing data were analyzed using HTSeqGenie (Pau and Reeder, 2012) in BioConductor (Huber et al., 2015) as follows: first, reads with low nucleotide qualities (70% of bases with quality <23) or rRNA and adapter contamination were removed. The reads that passed were then aligned to the reference genome GRCh37 using GSNAP (Wu and Nacu, 2010). Reads that were uniquely mapped were used for subsequent analysis. Differential expression analysis was performed using voom/limma (Law et al., 2014).

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Life sciences study design

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Sample size
No statistical methods were used to predetermine sample size. A minimum of 3 individual mice (biological replicates) were used for all major experiments. Sample size of each experiment reflects the number of biological replicates deemed necessary to adequately distinguish differences between genotype or condition and to ensure reproducibility of results. Additional details for each experiment is included in the corresponding figure legend.

Data exclusions
No data were excluded

Replication
Results from all experiments described were repeated in at least 2 (usually 3) independent experiments. All attempts of replication were successful.

Randomization
Mice of similar ages were used for all the experiments reported. Animals were allocated to groups based on their genotype or experimental treatment.

Blinding
Data acquisition and analysis was not blinded. All conclusions in this study were made based on statistical significance of the data.

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Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | Antibodies            |
| ☒   | Eukaryotic cell lines |
| ☒   | Palaeontology         |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |
| ☒   | Clinical data         |

Methods

| n/a | Involved in the study  |
|-----|------------------------|
| ☒   | ChIP-seq               |
| ☒   | Flow cytometry         |
| ☒   | MRI-based neuroimaging |

Antibodies

| Antibodies used |
|-----------------|
| Antibody (clone), conjugate, manufacturer, catalog#, lot #, (concentration) |
| αCD45 (30-F11), Pacific Blue, Biolegend, 103126, B253970, (1:500) |
| αCD45 (30-F11), PE Cy7, Biolegend, 103114, B243728/B163862 (1:800) |
| αCD31 (390), Pacific Blue, Biolegend, 102422, B182438, (1 :800) |
| αMadCAM-1 (MECA-367), Unlabeled, Biolegend, 120702, B215031 |
| αMadCAM-1 (MECA-367), AlexaFluor 488, Biolegend, 120708, B209791, (1 :100) |
| αMadCAM-1 (MECA-367), PE, Biolegend, 120710, B220914, (1:100) |
| αB220 (RA3-6B2), APC, Biolegend, 103212, B271008, (1:200) |
Validation

All antibodies used in this manuscript are from commercial sources and have been validated for the indicated species and application by the manufacturer. Descriptions of the validation approach used by each manufacturer are available at the following locations:

- Biolegend - https://www.biolegend.com/reproducibility
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Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

- C57BL/6J mice, VillinCre mice (021504), and Piezolfl/fl mice32 (029213) were purchased from Jackson Laboratories (Cambridge, Bar Harbor, MA, USA).
- Ccl19Cre mice were obtained from Dr. Burkhard Ludwig and have been previously described. (Chai et al, Immunity, 2013)
- Rosa26-SSB-BFP mice were generated in the laboratory and previously described.
- VillinCre mice were crossed to the Rosa26-SSB-BFP line to generate VillinCre-SSB-BFP mice. (Deng et al, Clin Exp Immunol, 2018)
- Ccl19Cre mice were crossed to the Piezolfl/fl mice to generate Ccl19-PickKO mice.

Mice were maintained under specific pathogen-free conditions in accordance with institutional and National Institute of Health guidelines and used at 6-8 weeks of age. For each of the experiments performed, age-matched mice of both sexes were analyzed.

Wild animals

This study did not involve wild animals.

Field-collected samples

This study did not involve samples collected from the field.

Ethics oversight

Experiments were approved by the Boston Children’s Hospital and Harvard Medical School institutional animal care and use committee in accordance with NIH guidelines for the humane treatment of animals.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

_confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.
Methodology

Sample preparation

| Single cell suspensions were prepared from freshly harvested tissues. Leukocytes and stromal cells were harvested from intestinal Peyer’s patches, inguinal lymph nodes, or mesenteric lymph nodes by enzymatic digestion (as described in the methods section), filtered through a 70um cell strainer, and resuspended in FACS buffer (PBS, 2% FBS, 1mM EDTA). Splenocytes were prepared by pressing the spleen through a 70um filter and lysing red blood cells. Single cell suspensions were treated with Fe block for 15 minutes on ice, and then stained with antibody for an additional 20 minutes on ice. Cells were analyzed immediately following preparation. |

Instrument

| Data acquisition was performed using a FACSCanto |
| Cell sorting was performed using a FACSARIA Special Order |

Software

| Data collection using FACS Diva |
| Data analysis using FlowJo (v10.3) |

Cell population abundance

| Cells were sorted for RNAseq analysis. Given the low abundance of the desired stromal cell fractions, only 1,000 cells were collected per sample. Purity was >98% |

Gating strategy

| Cell populations of interest were initially identified by cell size in a FSC-A vs. SSC-A plot, and single cells discriminated in a SSC-H vs. SSC-W plot. In some instances, cells were additionally gated using a live/dead marker (fixable viability dye efluo780, ebioscience). Lymphocytes were then identified as CD45+ while non-hematopoetic stromal cell populations were identified as CD45-. Lymphocyte populations were further identified along the following staining patterns: B cells (CD3-, B220+), T cells (CD3+, B220-), GC B cells (CD3-, B220+, GL7+, CD38-). Stromal cell populations were identified along the following staining patterns: FRCs (PDPN+, CD31-), LECs (PDPN+, CD31+), BECs (PDPN-, CD31+), HEV ECs (PDPN-, CD31+, MAdCAM1+). For quantification of absolute cell numbers, 50,000 CountBright beads (ThermoFisher Scientific) were added to each sample |

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.