Transcriptional programs of lymphoid tissue capillary and high endothelium reveal control mechanisms for lymphocyte homing

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Lymphocytes are recruited from blood by high-endothelial venules (HEVs). We performed transcriptomic analyses and identified molecular signatures that distinguish HEVs from capillary endothelium and that define tissue-specific HEV specialization. Capillaries expressed gene programs for vascular development. HEV-expressed genes showed enrichment for genes encoding molecules involved in immunological defense and lymphocyte migration. We identify capillary and HEV markers and candidate mechanisms for regulated recruitment of lymphocytes, including a lymph node HEV–selective transmembrane mucin; transcriptional control of functionally specialized carbohydrate ligands for lymphocyte L-selectin; HEV expression of molecules for transendothelial migration; and metabolic programs for lipid mediators of lymphocyte motility and chemotaxis. We also elucidate a carbohydrate-recognition pathway that targets B cells to intestinal lymphoid tissues, defining CD22 as a lectin-homing receptor for mucosal HEVs.

Blood vascular endothelial cells (BECs) in lymphoid tissues control the homeostatic homing of lymphocytes and recruitment of leukocytes during inflammation, regulate metabolite exchange and blood flow to meet the energy requirements of the immune response, and maintain vascular integrity and hemostasis. Such diverse functions require specialization of the endothelium. In lymphoid tissues, the capillary network is thought to be responsible mainly for solute and fluid exchange, whereas post-capillary high endothelial venules (HEVs) are specialized for the recruitment of lymphocytes1–3. In addition, HEVs display tissue specialization. HEV’s of skin-draining peripheral lymph nodes (PLNs) and gut-associated lymphoid tissue (GALT; this includes Peyer’s patches (PPs) and mesenteric lymph nodes (MLNs)) express tissue-specific vascular ‘addressins’, which are adhesion receptors that, together with chemokines, control the specificity of lymphocyte homing4. Despite the importance of vascular specialization to the function of the immune system, little is known about the transcriptional programs that define HEV specialization5. Studies have demonstrated the feasibility of isolating mouse lymphoid tissue endothelial cells (ECs) for transcriptional profiling and have characterized unique transcriptional programs of blood cells versus lymphatic ECs6.

Here we describe transcriptional programs of high endothelial cells (HECs) and capillary endothelial cells (CAP ECs) from PLNs, MLNs and gut-associated PPs. Our study defines transcriptional networks that discriminate capillary endothelium from high endothelium and identifies predicted determinants of HEV differentiation and regulators of HEV and capillary microvessel specialization. It also identifies gene-expression programs that define the tissue-specific specialization of HEVs, including mechanisms for the recruitment of B cells to GALT, and reveals unexpected tissue specialization of capillary endothelium as well. The results identify transcriptional and predicted metabolic, cytokine and growth-factor networks that may contribute to tissue and segmental control of the homing of lymphocytes into lymphoid tissues and to the regulation of local immune responses.

RESULTS

Transcriptional specialization of lymph node and PP BECs

We generated whole-genome expression profiles of lymphoid tissue BEC subsets using minor modifications of established protocols5. We sorted HECs from PLN BECs through the use of monoclonal antibody MECA-79 to peripheral lymph node addressin (PNAd), which comprises sulfated carbohydrate ligands for the lymphocyte-homing receptor L-selectin (CD62L). PP HECs were defined by monoclonal antibody MECA-367 to the mucosal vascular addressin MadCAM1, an immunoglobulin-family ligand for the gut lymphocyte-homing receptor α4β7. CAP ECs were defined by reactivity with MECA-99, an EC-specific antibody6 of unknown antigen specificity that distinguishes lymphoid tissue CAP ECs from HEVs (Fig. 1a,b).

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HEC versus CAP EC gene-expression signatures and pathways

To identify sources of variability in gene expression, we applied principal-component analysis to profiles of genes selected for different expression (difference of twofold between any pair of samples; \( P < 0.05 \) by one-way analysis of variance (ANOVA)) and with raw expression (EV) of >140 in 100% of replicates of at least one sample population. Numbers in parentheses indicate the proportion of total variability calculated for each principal component (PC). Each data point represents a biological replicate of cells sorted from tissues pooled from 10–14 mice (equal numbers of males and females). (d) Hierarchical clustering by correlation of samples of PLN, MLN and PP HECs and CAP ECs with the gene list defined in c, based on log2 normalized EVs (key). Each terminal branch represents results from a single microarray analysis of an independent biological replicate of sorted cells as described in e. * \( P < 0.05 \) and ** \( P < 0.01 \) (multiple bootstrap resampling). Data are representative of seven (PLN) or six (PP) independent experiments with samples pooled from 10–14 mice in each (a) or more than five independent experiments (b) or are from experiments with 12 independent groups of mice (10–14 mice per group; c, d).

We initially sought additional cell surface markers of lymphoid tissue endothelial specialization, both to confirm the identity of the sorted cells and to assess potential heterogeneity. We identified CD63, a tetraspanin protein linked to P-selectin function on activated ECs, as an HEV marker that uniformly and selectively decorated dissociated HECs and CAP ECs versus HEVs. Tissue-specific differences in gene expression dominated the second principal component (Fig. 1c). While specialization of lymph node versus gut-associated HEVs is well described in terms of vascular addressins, the principal-component analysis revealed robust tissue-specific differences in CAP EC transcriptomes as well. This suggested a previously unappreciated specialization of the capillary vasculature in PPs versus PLNs. MLNs are known to share features of both PLNs (for example, expression of PNAβ and most HEVs) and PPs (expression of MadCAM1 by subsets of MLN HEVs). Consistent with this, the transcriptional profiles of MLN HECs fell between those of their counterparts in PLNs and PPs. Clustering by Pearson’s correlation confirmed the significance of sample clusters that reflected tissue and segmental differences in gene expression (Fig. 1d).

HEC versus CAP EC gene-expression signatures and pathways

To define transcriptional signatures specific to HECs and CAP ECs, we compared HECs and CAP ECs from PLNs, MLNs and PPs. For each tissue, we identified genes expressed by HECs or CAP ECs (EV > 140) that had a difference in expression of at least 1.5-fold in HECs versus CAP ECs (Fig. 2a). We defined an HEC signature gene set of 799 genes whose expression was elevated in HECs in all three tissues and a CAP EC signature gene set of 642 genes whose expression was elevated in CAP ECs in all three tissues (Supplementary Table 1) and used these gene sets for gene-ontology (GO) term analyses and pathway analyses (reported below). We also identified 100 genes from the signature gene sets that differed in expression by at least fourfold in HECs versus CAP ECs (EV > 900) (Fig. 2b).

As expected, given the morphology and histochemical properties of HEVs, GO analyses of the HEC signature genes revealed enrichment for genes encoding molecules involved in Golgi and endoplasmic reticulum and generally in aspects of metabolism, notably including glycosylation, lipid and sterol metabolism (Fig. 3a). HEC signature genes also showed significant enrichment for genes encoding molecules associated with GO terms for defense, inflammatory response, chemokine activity and lymph node development, as well as molecules in the transcription factor NF-kB signaling pathway (Fig. 3a).
HEVs have key roles in the development of lymphoid tissues, including lymph nodes and PPs in perinatal life, as well as in tertiary lymphoid tissues in sites of chronic inflammation. Signaling by NF-κB through lipoxigenase is required for the maintenance of HEV’s in vivo, and tumor-necrosis factor (TNF) and Toll-like receptor ligands signal through NF-κB to induce vascular adhesion receptors and chemoattractants for the recruitment of leukocytes. Pathway analyses (with the Kyoto Encyclopedia of Genes and Genomes database and the Enrichr enrichment-analysis tool) confirmed enrichment for genes encoding molecules involved in glycan synthesis and metabolism and in sphingolipid metabolism (data not shown). As expected, HECs expressed Nr2f2, which encodes the master venous regulator NR2F2 (COUP-TFII) (Fig. 3b, bottom). This analysis did not reveal enrichment in the HEV signature group for genes encoding molecules assigned cardiovascular or endothelium-specific GO terms (data not shown). In contrast, GO terms related to endothelial development and angiogenesis featured prominently among CAP EC signature genes (Fig. 3a). CAP EC signature genes also showed enrichment for genes encoding molecules involved in pathways linked to vascular differentiation, including the Wnt, transforming growth factor-β and Notch signaling pathways (Fig. 3a).

Notably, CAP ECs expressed genes encoding molecules associated with arterial specification during embryonic vasculogenesis, including Notch4, Efnb2, Nrp1, Jag2, Dll4, Gja5, Kdr (Fig. 3b and Supplementary Table 1) and Hes1 (data not shown).
Figure 3  GO and pathway analyses of HEC and CAP EC signature gene sets.  (a) Selected GO terms and pathways (left margin) showing significant enrichment ($P < 0.05$, with use of the Benjamini-Yekutelli correction in GeneSpring software) in the signature gene sets reported above (Fig. 2a); order and box color are based on $-\log_{10}$ of the corrected $P$ value (key).  (b) Expression, in HECs versus CAP ECs, of genes belonging to various GO terms; large dots (with adjacent labels) indicate HEC or CAP EC signature genes (difference in expression of more than 1.5-fold in HECs versus CAP ECs (gray dashed lines, cutoff); $P < 0.05$); symbol colors indicate association with GO terms (key).  Results are presented as the average of all HEC samples (three to four samples each from PLNs, MLNs and PPs) or all CAP EC samples (two samples from MLNs and three samples from PLNs and PPs).  PDGF, platelet-derived growth factor; TGF-$\beta$, transforming growth factor-$\beta$.  (c) Immunofluorescence histology of PLNs from 8-week-old BALB/c mice, stained with anti-Nrp1 and MECA-99; PP sections showed a similar pattern (data not shown).  Scale bar, 100 $\mu$m.  (d) Immunofluorescence microscopy of PLNs from transgenic reporter mice with a gene encoding green fluorescent protein (GFP) knocked into the gene encoding Hes1, stained with anti-GFP (green) and MECA-99 (red); PP sections showed a similar pattern (data not shown).  Scale bar, 100 $\mu$m.  Data are representative of three independent experiments (c) or two independent experiments (d).
Immunofluorescence staining of tissue sections confirmed expression of Nrp1 (Fig. 3c) and Hev1 (Fig. 3d and Supplementary Fig. 1) by MECA-99® capillaries. In contrast, HECs expressed Nrp2f2, which (as noted above) encodes a venous regulator (Fig. 3b, bottom). As suggested by GO analysis, CAP ECs also had high and selective expression (relative to that in HECs) of several genes encoding molecules linked to angiogenesis, including Esım1 (which encodes ESM-1), Bgn (which encodes biglycan) (Supplementary Table 1) and several angiogenesis-associated G protein–coupled receptors (GPCRs) and their ligands, such as Cxcl12 and Cxcr4 (Fig. 3b). ESM-1 is involved in angiogenic sprouting but is also a secreted ligand for LFA-1 (integrin αβ2) and inhibitor of leukocyte adhesion;11 it may help prevent leukocyte arrest in capillaries. CAP ECs also expressed various growth factors and receptors (Fig. 3b). Genes encoding all three vascular endothelial growth factor (VEGF) receptors (Flt1, Flt4 and Kdr) and Vegfrc showed ‘preferential’ expression by CAP ECs, whereas Vegfrb had higher expression in HECs (Fig. 3b) and Vegfa was expressed by both HECs and CAP ECs (data not shown). The genes encoding aquaporin-1, aquaporin-7 and aquaporin-11, which regulate tissue fluid, glycerol and potentially CO2 exchange,12 had exclusive expression by CAP ECs (Aqp7 and Aqp11) or higher expression by CAP ECs than by HECs (Aqp1) (Fig. 2b and Supplementary Table 1). These results revealed transcriptional control of the anti-adhesive, angiogenic and transport properties of the capillary endothelium.

HEC signature genes included several encoding proteins involved in innate defense, such as components of the complement cascade (C1s, Cfb and the decay-accelerating factor CD55), PGRP-S (encoded by Pglyrp1), a pattern receptor for peptidoglycans of Gram-positive bacteria, and the hepcidin antimicrobial protein HEPC1 (encoded by H Amp) (Fig. 3b). HECs also had ‘preferential’ expression of genes encoding serpin A3N (Fig. 3b) and serpin A1C (Supplementary Table 1), which are inhibitors of the neutrophil proteases cathepsin G and elastase (as indicated by entries in the UniProt Consortium database). Neutrophils roll on HECs and are activated during extravasation when lymph nodes are inflamed; the presence of these inhibitors may prevent damage to ECs. Although CAP EC signatures tended to show enrichment for transcripts encoding molecules involved in angiogenesis, HECs had higher expression of the gene encoding LRG1, an HEV marker and regulator of transforming growth factor-β signaling in ECs linked to neovascularization13 (Supplementary Table 1). The gene encoding LYVE-1, a marker of lymphatic ECs, had higher expression by HECs than by CAP ECs (Fig. 2b and Supplementary Table 1), but its expression by BECs is much lower than that by lymphatic ECs. HEV signature genes encoding molecules involved in NF-κB signaling included the gene (Ubd) encoding ubiquitin-D (Fig. 2b and Supplementary Table 1), which facilities degradation of the NF-κB inhibitor IκB, and the EC-specific TNF family member vascular endothelial cell growth inhibitor (encoded by Tnfsf15; Fig. 3b), which activates NF-κB and serves as an autocrine inhibitor of endothelial growth and modulator of vascular homeostasis (as cited in an entry in the UniProt Consortium database).

Chemokines and cytokines and their receptors, and GPCRs
HECs as well as CAP ECs expressed genes encoding receptors for cytokines of the immune system (Fig. 4a). Genes encoding the interleukin 1 (IL-1) receptor (IL1r1) and several members of the TNF receptor family (Tnfrsf9, Tnfrsf11a, Relt and Eda2r) showed ‘preferential’ expression in HECs, while genes encoding the TNF receptor family members CD95 (Fas) and OPG (Tnfrsf11b) had higher expression in CAP ECs. Genes encoding the TNF receptor family member CD120a (Tnfrsf11a) and the receptor for lymphotixin-β (Llbr) had uniformly high expression in both HECs and CAP ECs. Genes encoding the cytokine receptors IL-3Ra (IL3ra) and GM-CSFRa (Csfr2a) and their common β-chain coreceptor (Csfr2b) were expressed by HECs and CAP ECs. The gene encoding the common γ-chain (Il2g) had high and somewhat ‘preferential’ expression by HECs. While HECs and CAP ECs had similar expression of genes encoding receptors for type 1 interferons and interferon-γ (IFN-γ), HECs had higher expression of the gene encoding IFN-γ receptor 2 (Ifngr2) than did CAP ECs. Genes encoding the receptors for IL-27 (Il27ra), IL-11 (Il11ra), oncostatin M (OsMr) and leukocyte-inhibitory factor (Ljfr) and their common partner chain gp130 (Il6st) were expressed by HECs; expression of Il27ra and Il6st was HEC selective. Notably, CAP ECs constitutively expressed the gene encoding for IL-6, which is cytotoxic for ECs14, but HECs did not, whereas the gene encoding its receptor IL-6R0 (Il6ra) was expressed in both HECs and CAP ECs. Thus, HECs and CAP ECs had both distinct and overlapping expression of receptors for homeostatic and inflammatory cytokines.

In the multistep process of lymphocyte recruitment, rolling lymphocytes sample the EC surface for chemokines that can trigger integrin-dependent arrest. Chemokines involved in the process can be expressed by HECs or can be delivered to ECs from surrounding tissues or lymph; they can be presented on the luminal surface of ECs by heparan sulfate proteoglycans, which are glycosaminoglycans that bind chemokines, growth factors and other factors.15 We identified chemokines and receptors expressed in HECs or CAP ECs (EV > 140) (Fig. 4a). We confirmed high HEC-specific expression of the gene encoding CCL21, which triggers lymphocyte arrest on HEV3,4,16. Unexpectedly, HECs also had constitutive expression of genes encoding CXCL10 and CXCL11; these chemokines function as ligands for the inflammatory trafficking receptor CXCR3 (refs. 17,18). Although CXCL12 and CXCL13 are displayed by HEVs and participate in the recruitment of B cells to PP3,17, HECs had low expression of genes encoding these chemokines; therefore, they are probably derived from surrounding stromal sources. Such tissue-derived chemokines, as well as chemokines that arrive in lymph, can be transported from the abluminal to luminal surface of venular ECs by Darc (encoded by Ackr1), a unique nonsignaling chemokine receptor specialized for this function.18 Ackr1 had high expression by HECs but not by CAP ECs in our samples. HECs also expressed Ackr2, which encodes the scavenger receptor Cbp2b (D6) that functions to internalize and clear chemokines from the cell surface.18 Genes encoding several hepatic sulfate proteoglycan core proteins had different expression by HECs and CAP ECs as well. Differences in the expression of these proteins, as well as EC subset–selective modifications of their heparan sulfate side chains, might regulate chemokine display. Together these results demonstrated transcriptional control of the expression of not only EC chemokines but also molecules involved in endothelial transport, presentation and degradation of chemokines.

Chemokines and other GPCR ligands also regulate endothelial responses19. Genes encoding the chemokine CXCL12 and its receptor CXCR4 showed selective expression by CAP ECs (Fig. 4a), where they may regulate endothelial migration and angiogenesis. CAP ECs also had constitutive expression of Cx3Cl1, which encodes the transmembrane chemokine fractalkine. Fractalkine has constitutive expression by arterial endothelium, and its expression is reportedly induced in vivo by TNF in capillary and arterial endothelium but not venous endothelium20, and it can mediate angiogenesis.21 The gene encoding the long amino-terminal GPCR CD97, which may regulate strengthening of the adherens junction and induce angiogenesis, showed selective expression by CAP ECs, as did the gene encoding...
Figure 4 Expression of selected cytokines and chemokines and their receptors, integrins, GPCRs, transcription factors, and mucin domain–containing or immunoglobulin domain–containing adhesion receptors in HECs and CAP ECs of lymphoid tissues. (a) Expression of selected gene families, showing genes with an EV of >140 in any sample (box color, blue font (left margin) and numbers along right margin, as in Fig. 2b). (b) Expression of genes encoding selected immunoglobulin-family, mucin-domain, selectin and other adhesion receptors, or selected enzymes linked to phospholipid and oxysterol metabolism (above plots). Each symbol represents an individual biological replicate; small horizontal lines indicate the mean (and s.e.m.). (c) Immunofluorescence microscopy of PLN cryosections stained with anti-Parm1 (red) and MECA-79 (green); insets (bottom right) show detection of Parm1 (yellow arrows) on the HEV surface (2× enlargement). Scale bar, 100 μm. Data are representative of experiments as in Figures 1 and 2b (a; mean of replicates) or three independent experiments with tissues from one mouse in each (c).
the endothelin receptor ETb-R (Ednrb). This receptor is involved in the generation of nitric oxide, which promotes microcirculation. The gene encoding the CXCR3 ligands CXCL10 and CXCL11, which are angiostatic,17,18, were expressed by HECs. Together these results showed that HECs and CAP ECs had different expression of genes encoding an array of ligands and receptors that can mediate communication with the local environment to control leukocyte recruitment and regulate segmental EC responses.

### Adhesion, mucin and enzyme receptors for homing

Several sialomucins have been shown to act as acceptors of L-selectin-binding glycotopes that mediate tethering and rolling of blood-borne leukocytes on HEVs. The gene encoding the adhesion molecule CD34 (Cd34) had high expression in both capillaries and HECs, whereas transcripts encoding the adhesion molecule GlyCAM-1 (Glycam1) had ‘preferential’ expression in HECs (Fig. 4b). Podocalyxin (encoded by Podxl) can accept L-selectin-binding glycotopes and is reportedly expressed by HEVs2,24, but our data revealed ‘preferential’ expression of Podxl in CAP ECs (Fig. 4b), which suggested that the role of podocalyxin in cell repulsion and in the formation of EC tubes25 may be more important. Nepmucin (encoded by Cd300lg), which presents L-selectin ligands and also binds lymphocytes via its amino-terminal V-type immunoglobulin domain, is displayed by PLN HECs but not by PP HECs24, which correlates with Cd300lg expression, as shown here (Fig. 4b). However, Cd300lg and Emcn (which encodes endomucin) had a similar expression pattern, but both had somewhat higher expression by CAP ECs than by HECs (Fig. 4b). Our gene profiling also revealed selective expression of Parm1 by HECs (Fig. 4b). Immunofluorescence histology confirmed expression by HECs of its product PARM-1 (ref. 25) (Fig. 4c), a mucin not previously reported to be present on HEVs, and immunoblot analysis demonstrated decoration of PARM-1 by PNAd glycotypes, as indicated by MECA-79 reactivity (Supplementary Fig. 2).

Genes encoding the β2 integrin ligands ICAM-1 (which mediates arrest of rolling lymphocytes on HEVs) and ICAM-2 were expressed by lymphoid HECs and CAP ECs (Fig. 4b). The gene encoding the αvβ1 integrin ligand VCAM-1 had high expression (EV = −1.000) in all lymphoid EC subsets as well (Fig. 4b), even though this vascular adhesion molecule is expressed only infrequently and weakly at the protein level by ECs in lymph nodes or PPs. Similarly, vascular E- and P-selectin, although difficult to detect on resting HEVs (data not shown), were well represented in HECs at the RNA level (Fig. 4b). Although we cannot exclude the possibility of the upregulation of genes during the isolation of ECs, these results suggested that expression of VCAM-1 and the vascular selectins might be regulated post-transcriptionally in BECs in vivo.

Among other genes encoding molecules linked to lymphocyte homing via HEVs, Stab1 (which encodes the common lymphatic endothelial and vascular receptor CLEVER-1)26 had uniform expression by CAP ECs and HECs (Fig. 4b). Aoc3 (which encodes the inducible vascular adhesion protein VAP1)27 had high expression by CAP ECs but not by HECs in our samples (Fig. 4b); although VAP1 constitutively decorates HECs in humans27 (M.D.L. and E.C.B., data not shown), the lack of Aoc3 expression in HECs in our samples would suggest that HEV-associated VAP1 immunostaining observed in resting mouse lymph nodes might have been on pericytes.

### Genes linked to lipid mediators of lymphocyte migration

HECs expressed genes encoding molecules involved in the synthesis and transport of lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), which are lipid mediators of lymphocyte motility and chemotaxis (Fig. 4b). HECs as well as CAP ECs expressed the gene encoding autotaxin (Enpp2), which is functionally important for the generation of LPA and the recruitment of lymphocytes via HEV-24,28. Genes encoding the sphingosine kinase SK1 (Sphk1) and the acylsphingosine deacylase Asah2 (Asah2), which are involved in the synthesis of S1P, showed ‘preferential’ expression by HECs. Asah2 generates sphingosine from N-acylsphingosine, and SK1 phosphorylates sphingosine to generate S1P. S1P potently stimulates lymphocyte motility and, through S1P1 (encoded by S1pr1), the T cell receptor for S1P, enhances integrin-dependent arrest of T cells in PLNs but not PPs29. That tissue difference in the activation of T cell arrest by S1P may relate to the observed higher expression of Sphk1 in PLN HECs than in PP HECs (1.5-fold higher in PLN HECs than in PP HECs; P < 0.05). SK1 is an intracellular enzyme, but HECs and CAP ECs also expressed Spns2, which encodes the S1P transporter required for S1P’s support of lymphocyte exit from bone marrow and thymus. Autocrine production or exogenous sources of S1P and LPA probably affect ECs directly, as well, since BECs had high expression of S1pr1 and both Lpar4 and Lpar6 (which encode LPA receptors 4 and 6, respectively) (data not shown). Lpar6 had ‘preferential’ expression by CAP ECs (Fig. 4a).

HECs had high expression of the gene encoding cholesterol 25-hydroxylase (Ch25h), which synthesizes 25-hydroxycholesterol (25-OHC), but CAP ECs did not (Fig. 4b). PP HECs and, to a lesser extent, PLN HECs also expressed the gene encoding cholesterol 27-hydroxylase (Cyp27a1), which generates 27-hydroxycholesterol (Fig. 4b). These sterols are the immediate precursors of potent chemotactic ligands for the lymphocyte receptor EB12 (encoded by Gpr183)30,31. However, HECs also expressed the gene encoding the hydroxysteroid dehydrogenase HS3B7, which degrades EB12 ligands (Fig. 4b), but they lacked the enzyme CYP7B1 required for generation of the active ligands (data not shown).

### Differently expressed transcription factors

BEC subsets in lymphoid tissues had different expression of genes encoding an array of transcription factors (Fig. 4a), including ligand-activated transcription factors (for example, androgen receptor (encoded by Ar), which was expressed by HECs, and PPAR-γ (encoded by Pparg) and the retinoic acid receptor RARγ (encoded by Rarg), which had higher expression by CAP ECs), transcription factors linked to cardiovascular development (for example, Sox17, Msx1, Id1, Id3, JunB and Moeox2), and transcription factors involved in regionalization or digestive system development (for example, FoxP4, Hlx, Hoxd8, Lhx2, Egfr2, Tcf7l1 and Meis2). Notably, PP HECs and PP CAP ECs (but not PLN BECs) both expressed the gene encoding NKX2-3, a homeobox transcription factor involved in development of the gastrointestinal tract that is required for the expression of MadCAM1 by ECs in vivo32. These factors may help control the segmental and tissue specialization of GALT versus PLN HEVs.

### Tissue-specific specialization of HECs

To assess the tissue-specific specialization of HECs, we focused on genes expressed differently by PLN HEVs versus PP HEVs. We identified the signature genes of PLN HEVs or PP HEVs as those with an EV of >140 and a difference in expression of 1.5-fold or more (P < 0.05) in PLN HEVs relative to their expression in PP HEVs, with 1.5-fold (or more) higher expression by PLN HECs or PP HECs than by CAP ECs of PLNs, MLNs and PPs, and with 1.5-fold (or more) higher expression by PLN HECs or PP HECs than by naive or memory T cells. We used the resulting 150 signature genes of PLN HEVs and 48 signature genes of PP HEVs for GO-term analyses (Supplementary
expression of the gene encoding CD73 (Nt5e) (Fig. 4b), which is a rate-limiting enzyme (ecto-5'-nucleotidase) involved in conversion of extracellular proinflammatory ADP and ATP into adenosine. Through the generation of adenosine and resultant adenosine signaling, endothelial CD73 has anti-inflammatory and tissue-protective roles and regulates the recruitment of lymphocytes via HEVs and the recruitment of leukocytes in models of inflammation37.

GO term analysis of the PP HEV signature genes revealed enrichment for genes encoding molecules involved in ‘defense response’ (Fig. 4c), including HDAC9 (Fig. 5c), and those encoding the chemokines CXCL10 and CXCL11 (Fig. 5a), which are classically upregulated in inflammation and recruit activated subsets of T cells as well as monocytes. Although their gene expression by HEVs has not been reported, CXCL10 can decorate HEVs during inflammation, which has led to the suggestion that CXCL10 on HEVs is derived from lymph or stromal cells13; our results suggested that,
Glycoproteins on the endothelial surface undergo carbohydrate modifications that control lymphocyte adhesion in PPs than in resting PLNs. Part the greater steady-state inflammatory and immunological stimulation in PPs than in PLNs might be endogenously expressed by HECs as well. HDAC9 mediates proinflammatory epigenetic changes in cells of the immune system and also regulates angiogenesis. Notably, genes with selective expression in PP HEVs that encode molecules associated with ‘defence response’ also included Scd1 (Fig. 5a), which encodes a fatty acid desaturase that is induced by stress and maintains EC function. PLN HECs and PP HECs also differed in their expression of genes linked to GO terms for the biosynthesis of steroids, lipids and prostanoids (Fig. 5c). In addition to the differences noted above in the expression of Ptgsl (which encodes COX-1), the expression of Skco2a1 (which encodes the prostaglandin transporter PGT) was fourfold higher in PP HEVs than in PLN HEVs and was twofold higher in PP CAP ECs than in PLN CAP ECs (data not shown), consistent with regional differences in eicosanoid biology. PP HEVs also expressed the gene encoding the corticosteroid dehydrogenase 11β-HSD2 (Hsd11b2), which metabolizes intracellular cortisol, converting it to the inactive metabolite cortisone, but PLN HEVs did not express this gene (Fig. 5a). The gene encoding Gpr126, an adhesion GPCR, was expressed exclusively in PP HECs and one MLN HEC preparation (Fig. 5a). Although Gpr126 has not been detected previously in ECs in vivo, perhaps due to its highly restricted expression, it has been linked to cardiovascular development and its expression is upregulated by lipopolysaccharide in human umbilical vein ECs. Together these results suggested that gene expression in PP HEVs reflects in part the greater steady-state inflammatory and immunological stimulation in PPs than in resting PLNs.

Transcriptional control of L-selectin-binding glycoproteins
Glycoproteins on the endothelial surface undergo carbohydrate modifications that control lymphocyte adhesion, as well as interactions
In contrast to the genes encoding enzymes involved in NAcLac synthesis, genes encoding most enzymes responsible for terminal modifications required for L-selectin binding had significantly higher expression in PLN HEVs than in PP HEVs (a difference in expression of at least 1.5-fold; \( P < 0.05 \); Fig. 6b). These included Chst2 and Chst4, which encode HEV carbohydrate (N-acetylgalactosamine-6-O) sulfotransferases.\(^{13,37}\) The expression of Chst4 was over tenfold higher in PLN HEVs than in PP HEVs. Chst2 had high expression by all HEVs, but displayed significant selectivity for PLN HEVs as well. Chst4\(^{-/-}\) mice have a more severe defect in the homing of lymphocytes to PLNs than do Chst2\(^{-/-}\) mice, and double-deficient Chst2\(^{-/-}\)/Chst4\(^{-/-}\) mice display only minimal residual L-selectin-dependent lymphocyte rolling in PLN HEVs.\(^{16,37}\) As reported, Chst2 was also expressed by PLN HEVs and PP HEVs (but had little if at any expression by CAP ECs) (Supplementary Table 1); this gene encodes keratan sulfate galactose-6 sulfotransferase, which generates 6-sulfo-SLeX in culture models but does not contribute detectably to L-selectin-mediated homing.\(^{32}\) Genes encoding enzymes linked to the addition of terminal sialic acid and fucose residues of SLeX (St3gal4 and Fut7, respectively) also had significantly higher expression in PLN HEVs than in PP HEVs (\( P < 0.05 \); Fig. 6b), although the difference in expression was small compared with that of Chst4. St3gal4\(^{-/-}\) mice have deficient L-selectin-mediated lymphocyte rolling in inflamed extravascular venules but normal interactions of lymphocytes with HEVs.\(^{36,38}\) However, HEVs expressed genes encoding each of the other known \( \beta \)-galactoside \( \alpha,2,3 \)-sialyltransferases as well (St3gal1, St3gal2, St3gal3, St3gal5 and St3gal6; data not shown). St3gal6 had particularly high expression by HEVs, although its expression was equal in PLNs and PPs (Fig. 6b). The gene encoding CMAH (cytidine monophosphate-N-acetylneuraminic acid hydroxylase), an enzyme that converts the terminal sialic acid of L-selectin ligands to N-glycolyneuraminic acid,\(^{38}\) had 1.7-fold higher expression by HEVs in PLNs than those in PPs (Fig. 6b). Genes encoding HEV UDP-fucose and sulfate transporters (Slc35c1 and Slc26a2), the latter reported in human tonsil HEVs,\(^{39}\) also had slightly higher expression by PLN HEVs (Fig. 6b). HEVs actively take up sulfate from the environment\(^{40}\) and may import UDP-fucose as well to enhance substrates for the synthesis of 6-sulfo-SLex. Overall, the data suggested that genes encoding key enzymes involved in the terminal steps of L-selectin ligand synthesis were regulated in a tissue-selective fashion in HEVs, as were genes encoding transporters that provide UDP-fucose and sulfate as enzyme substrates.

CAP ECs had reduced expression of genes encoding each of the regulated L-selectin ligands that distinguish PLN HEVs from PP HEVs (Fig. 6b). However, CAP ECs were also deficient in expression of Gcnt1, which encodes the core 2 branching GlcNAc transferase (Fig. 6a,b). Branching core 1 or core 2 glycans strengthen L-selectin-mediated rolling via enhanced valency.\(^{35}\) Diminished core 2 branching may limit the potential for aberrant lymphocyte interactions in capillaries. CAP ECs also expressed genes encoding glycosyltransferases that directly inhibit SLeX synthesis, including St3gal1, whose expression was higher in CAP ECs than in HEVs in both PLNs and PPs (Fig. 6b). The glycosyltransferase encoded by St3gal1 caps the proximal galactosyl-\( \beta,1,3 \) N-acetylgalactosamine of growing core 1 O-glycans and thus prevents the synthesis of core 1 or core 2 selectin ligands. Indeed, deficiency in this enzyme leads to enhanced production of L-selectin ligands by ECs and enhanced lymphocyte adhesion.\(^{36}\) CAP ECs also expressed genes encoding \( \alpha,2,8 \)-sialyltransferases, including St8sia4 (Supplementary Table 1), whose product modifies N-glycans with anti-adhesive sialic acid polymers in the nervous system.\(^{41}\) Together these results suggested important role for transcriptional programs in the segmental as well as tissue-selective adhesive properties of EC glycoconjugates.

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**Figure 7** Selective PP HEC expression of St6gal1 confers recognition of PP HECs by CD22 and CD22-mediated homing of B cells to GALT. (a) Structure of 6′-sialyl-LacNac, the minimal recognition determinant for SNA lectin, and possible structures of high-affinity PP HEV CD22 ligands (NeuGcαα-6Galβ1-4GlcNAc or sulfated NeuGcαα-6Galβ1-4(S)GlcNAc); black italic font indicates genes encoding enzymes involved; sulfate is in gray, since the existence of sulfated CD22 ligands in rodents is speculative. Number adjacent to gray arrow indicates difference in expression of St6gal1 in PP HEVs versus PLN HEVs. (b) Expression of St6gal1 in HECs and CAP ECs from the PLNs, MLNs and PPs of BALB/c mice (left), and flow cytometry of HECS from BALB/c mice, stained with SNA lectin and CellTracker Violet and were injected together into the host mice; cells isolated from the host mice were stained with anti-CD3 (to identify T cells), anti-CD19 and antibody to immunoglobulin D (IgD) (to identify B cells) for quantification of short-term (1.5-h) homing of B cells and T cells. Results are presented as the relative localization ratio (RLR), a measure of the efficiency of homing relative to that of wild-type CD3* T cells, set as 1. Similar results were obtained with CD45 allotypic markers to monitor the recruitment of B cells and T cells (data not shown; one experiment). * \( P < 0.01 \), versus homing of wild-type B cells into PPs of wild-type recipients (two-sided paired \( t \)-test). Data are from two independent experiments with tissues pooled from four mice in each (b, right) or are pooled from four independent assays in two independent experiments (c; mean and s.e.m. of four biological replicates).
To correlate transcriptional profiles with cell surface expression, we used antibodies to relevant glycoproteins\textsuperscript{37,42,43} (Fig. 6c). The antibody HECA-452 recognizes sialic acid– and fucose-dependent but sulfate-independent SLEx-related epitopes\textsuperscript{43}. The antibody MECA-79 recognizes peripheral addressin 6-sulfo-SLeX on core 1 O-glycans but not on core 2 O-glycans; recognition is dependent on sulfate but not dependent on sialic acid\textsuperscript{37}. The antibody S2 recognizes 6-sulfo-SLeX and 6-sulfo-N-acetyllactosamine on O- and N-glycans\textsuperscript{42,43}. S2 stained PLN HECs much more brightly (approximately tenfold more, by flow cytometry) than PP HECs, although both were positive (Fig. 6c,d). MECA-79 stained PLN HEVs, but the surface of PP HECs was essentially negative for MECA-79 staining (Fig. 6c). Immunohistochemical studies show abluminal staining but not luminal staining of PP HEVs with MECA-79 (ref. 1) (Fig. 6c). Our data raised the possibility that this abluminal MECA-79 reactivity was derived from pericytes rather than from HECs themselves and indicated that most PP HEV 6-sulfo-SLeX glycotypes were on core 2 O- or N-glycans. Consistent with predictions made on the basis of gene expression, the sulfate-independent SLEx epitopes recognized by HECA-452 decorated HEVs in both PPs and PLNs and were only two- to threefold more abundant on PLN HECs than PP HECs (Fig. 6c). CAP stained poorly with all three monoclonal antibodies (data not shown). The correlation of carbohydrate epitopes with expression patterns of genes encoding glycosyltransferases and sulfotransferases suggested that transcriptional control mechanisms specify the segmental (capillary versus HEV) expression and tissue-specific specialization of modified glycans that control L-selectin interactions.

**St6gal1 expression controls homing of B cells to PPs**

In contrast to genes encoding molecules responsible for L-selectin ligand synthesis, the gene St6gal1, which encodes the enzyme β-galactoside α-2,6-sialyltransferase 1 (ST6Gal I) had ‘preferential’ expression by PP HEVs (Fig. 6b, top, Fig. 7a, and Fig. 7b, left). It had moderate expression by MLN HEVs but low expression by PLN HEVs and by CAP ECs in all tissues (Fig. 7b, left). ST6Gal I is the sole enzyme outside the nervous system that adds sialic acid in α2,6 linkage to N-acetyllactosamine (LacNAc) to form Siaα2-6Galβ1-4GlcNAc (6′-sialyl-LacNAc) (Fig. 7a) and terminate N- and O-linked glycan cores\textsuperscript{44}. This terminal modification has not been reported on LeX and is believed to be mutually exclusive with the fucosylation required for generation of functional SLEx\textsuperscript{45}; thus, it may contribute to diminished binding of L-selectin in PPs. 6′-sialyl-LacNAc is recognized by the *Sambucus nigra* (SNA) lectin, and flow cytometry of cells stained with SNA confirmed selective display of α2,6-linked sialic acid by PP HEVs (Fig. 7b, right).

Moreover, ST6Gal I generates functional ligands for the B cell lectin CD22 (Siglec-2)\textsuperscript{38,46}, which in the mouse binds 6′-sialyl-LacNAc, with N-glycolyneuraminic acid (NeuGc) as the sialic acid as a ‘preferred’ ligand (NeuGcα2-6Galβ1-4GlcNAc)\textsuperscript{38,47}. The conversion of CMP-N-acetylneuraminic acid (NeuAc) to CMP-NeuGc is carried out by ST6gal1 (Fig. 6d). We analyzed the transcriptomes of lymphoid tissue HEV and capillary endothelium from lymph nodes and GALT, extending published pioneering studies of gene expression in mouse lymph node and human tonsillar HEVs\textsuperscript{48}. Our results have identified transcriptional networks that accompany EC specialization and have revealed the expression of cytokine receptors, GPCRs and growth factor receptors that allow CAP ECs and HECs to interpret signals from surrounding immune and stromal cells. They have also defined tissue-specific gene signatures for HEVs, revealing a central role for transcriptional mechanisms in the segmental and tissue-specific expression of endothelial determinants for the recruitment of lymphocytes.

**DISCUSSION**

We analyzed the transcriptomes of lymphoid tissue HEV and capillary endothelium from lymph nodes and GALT, extending published pioneering studies of gene expression in mouse lymph node and human tonsillar HEVs\textsuperscript{48}. Our results have identified transcriptional networks that accompany EC specialization and have revealed the expression of cytokine receptors, GPCRs and growth factor receptors that allow CAP ECs and HECs to interpret signals from surrounding immune and stromal cells. They have also defined tissue-specific gene signatures for HEVs, revealing a central role for transcriptional mechanisms in the segmental and tissue-specific expression of endothelial determinants for the recruitment of lymphocytes.

Our results have shown that lymphoid tissue capillaries express genes encoding molecules that directly vascular development, and share surface antigens with arterial ECs. Although this was not discussed here in detail, capillaries also displayed an unexpected tissue specialization in gene expression, including unique tissue-selective CAP EC–specific genes and genes shared with HECs in the same tissue. For example, both CAP ECs and HECs in GALT expressed NKX2-3, a transcription factor required for MAdCAM1 expression\textsuperscript{42}. Finally, CAP ECs were characterized by anti-adhesive transcriptional programs, expressing (for example) the soluble (decoy) LFA-1 ligand ESM-1, as well as glycosylation programs predicted to preclude the synthesis of SLEx-related ligands for leukocyte tethering.

The gene signatures of HECs revealed active metabolism and expression of molecules involved in defense and immunity, and confirmed transcriptional control of many known molecular pathways for lymphocyte interaction and recruitment. Consistent with published studies, HECs ‘preferentially’ expressed the gene encoding CCL21, which is involved in the recruitment of lymphocytes into lymphoid tissues, and GALT HECs had high expression of the gene encoding MAdCAM1, but PLN HECs did not.

Blood-borne lymphocytes initiate interactions with HEVs through L-selectin-mediated ‘tethering’ and rolling\textsuperscript{1,4,49}. The patterns of expression of genes encoding glycosyltransferases and sulfotransferases that control the synthesis of L-selectin ligands indicated a transcriptional basis not only for the HEV selectivity of L-selectin binding but also for fine control of the nature of L-selectin ligands. The genes expressed by PLN HEVs encoded products predicted to allow synthesis of core 1 as well as core 2 branching biantennary ligands for L-selectin. Consistent with that, PLN HEVs showed intense staining with MEC-79, the core 1–specific antibody to PNA. The generation of biantennary 6-sulfo-SLeX ligands may contribute to the high-avidity, low-velocity, L-selectin-mediated rolling of lymphocytes in PLN HEVs. In contrast, PP HEVs showed...
induces functional CD22 binding, together these features correlated with the greatly reduced reactivity of PP HECs with the S2 antibody to 6-sulfo-SLeX and lack of reactivity with MEGA-79, the antibody to PNAd that recognizes only core 1 L-selectin binding carbohydrates but not their core 2 counterparts. On the other hand, PP HECs stained only two- to threefold less well than PLN HECs with antibody HECA-452, which binds low-affinity L-selectin ligands as well as high-affinity L-selectin ligands, including nonsulfated SLeX. Reduced expression of high-affinity L-selectin ligands, combined with lack of the enhanced valency provided by biantennary 6-sulfo-SLeX-capped glycans, probably explains the less-avid (high-velocity) rolling mediated by L-selectin in PP HEVs4. Such ’loose’ rolling is critical to the specificity of the homing of lymphocytes to PP, since it does not slow cells sufficiently to allow chemokine activation of lymphocyte arrest and thus enforces a requirement for additional ‘braking’ mediated by interaction of the integrin α4β7 with MadCAM1 (refs. 4, 49).

Selective expression of St6gal1 in PP HEVs led us to identify a vascular addressin for the targeting of B cells to GALT. B cells home efficiently to PP in support of mucosal humoral immunity, while they home less well to PLNs than T cells do48. In short-term homing assays, St6gal1−/− mouse PPbs were defective at recruiting B cells from the blood. St6GalI generates ligands for B cell–expressed CD22 (Siglec2)38, and we showed that a CD22–immunoglobulin Fc chimera selectively bound PP HEVs. Moreover, CD22−deficient B cells showed reduced homing to wild-type PPs. These findings reveal a role for CD22 and ST6Gal I–dependent CD22 ligands in the homing of B cells to PPs. The known ‘preferred’ ligand for mouse CD22 is NeuGcα2-6LacNAc, and PP HECS expressed transcripts encoding enzymes required for its synthesis, including CMAH, which generates NeuGc from NeuAc. Humans lack CMAH, and human CD22 binds 6-sulfono NeuAcα2-6LacNAc as a ‘preferred’ ligand. While the specific structures of CD22 ligands on PP HEVs remain to be determined, they are probably generated by the combined actions of ST6GalI, CMAH and potentially HEV sulfotransferases; in addition to NeuGcα2-6LacNAc, 6-sulfo-NeuAcα2-6LacNAc is a likely candidate. Notably, CD22 contributes to the accumulation of mature recirculating B cells in bone marrow in which sinusoidal ECs express unknown CD22 ligands50; cytokine activation of ECs in vitro induces functional CD22 binding51, and human HEVs stain with an antibody to the CD22 ligand 6-sulfo-NeuAcα2-6LacNAc43, which has led to proposals that CD22 can function as a lymphocyte trafficking receptor. Our studies directly confirmed the ability of CD22 to mediate lymphocyte homing in vivo and defined a selective role for CD22 in the homing of B cells to GALT. CD22 is the first immunoglobulin family member to our knowledge shown to function as a lymphocyte-homing receptor through the recognition of ECs via the immunoglobulin domain. Other members of the immunoglobulin family displayed by leukocytes, in particular other Siglec proteins, should now be considered candidate receptors for endothelial recognition and leukocyte trafficking.

Our results also showed HEV expression of molecules linked to leukocyte-vascular interactions but not previously associated with high endothelium. BST-1, which is linked to neutrophil diapedesis in culture models33, had different expression by PLN HEVs versus PP HEVs, which suggests a role in tissue selective lymphocyte-HEV interactions. CD63 is required for the exocytosis of granules (Weibel Palade bodies) and for P-selectin expression following the activation of ECs7. HEV expression of CD63 suggests a potential role in lymphocyte-HEV interactions as well. The chemokine-scavenger receptor Ackr2, which is expressed by lymphatic endothelium and binds and internalizes inflammatory chemokines but not homeostatic chemokines to facilitate resolution of inflammation, was also expressed by HEVs, which suggests it may also limit inflammatory chemokine presentation by HEVs. Our analyses also identified B4GALT5 and B4GALT6 as additional candidate HEV glycosyltransferases for the synthesis of L-selectin ligands and revealed segmental and tissue-selective expression of sulfate and UDP-fucose transporters involved.

HEVs also expressed genes encoding enzymes for the metabolism of diverse lipid mediators, including eicosanoids, LPA and sphingosines linked to both vascular cell function and the function of cells of the immune system. In the context of lymphocyte migration, studies of S1P have focused mainly on its role in the exit of lymphocytes from lymphoid tissues into lymph. However, S1pr1 expression by lymphocytes contributes to interactions with PLN HEVs (but not PP HEVs)80, an observation that correlates with the higher expression of Sphki1 and Asah2 in PLN HEVs and suggests a role for local S1P production in homing. Autocrine synthesis of S1P may also have unique effects on HEVs: while plasma S1P supports the integrity and barrier function of ECs, intracellular S1P or overexpression of Sphki1 (which encodes the sphenogines kinase SK1) in ECs reduces cell proliferation and loosens or disrupts cell-cell junctions52, features arguably characteristic of HEVs. Elucidation of the importance of autocrine HEV expression of S1P will require targeted genetic manipulation of S1P metabolism. Consistent with published studies24, 28, HEVs (and, unexpectedly, also CAP ECs) had abundant expression of transcripts encoding autotaxin, which generates LPA locally and contributes to lymphocyte recruitment via HEVs.

HECs had high expression of transcripts encoding cholesterol 25-hydroxylase, which synthesizes 25-OHC, a sterol involved in lipid metabolism and immunological activation53. 25-OHC is the immediate precursor of 7α, 25OH Ch, the most potent known agonist for the lymphocyte and dendritic cell chemoattractant receptor EB2 (Gpr183). The 7-hydroxylase CYP7B1, which is required for generation of the active attractant, is expressed by lymphoid stromal fibroblastic reticular cells5. HECs also expressed the gene encoding the enzyme that degrades EB2 attractants, which could prevent stroma-derived EB2 agonists from reaching the vascular lumen. On the other hand, if enzyme activities correlate with gene expression, the transcellular metabolism predicted, with the generation of 25-OHC and degradation of stromal cell–derived 7α, 25OH CH by HECs, could establish a steep gradient of the agonist to attract EB2 (Gpr183)–expressing lymphocytes and dendritic cells away from HEVs and into the surrounding tissue. The role of EB2 (Gpr183) in lymphocyte recruitment via HEVs has not been examined.

Mucins have important roles as acceptors of glycotopes for lymphocyte interaction and repulsion. Our data have shown that the genes encoding CD34, podocalyxin, GlyCAM-1 and MadCAM1 were expressed in all ECs or selectively in capillaries, HEVs or GALT HEVs, respectively, which correlated with their reported protein expression. While their function as pro- or anti-adhesive factors depends on the nature of their carbohydrate modifications, their EC substrate–specific expression would suggest that mucins might have specialized roles in vivo, perhaps related to differences in glycosyltransferase substrate preferences. In addition to previously described mucins, we have identified PARM-1 as a previously unknown HEV-specific mucin that was ‘preferentially’ expressed in PLNs and have shown that it
was decorated by PNAd glycoproteins and thus probably contributes to L-selectin-mediated homing as well.

Not all gene expression in BECs correlated with protein expression. As not all genes, Vcam1 and genes encoding E- and P-selectin had high expression in HECs, and Stab1 had high expression in all BECs, while the adhesion receptors they encode are displayed minimally or undetectably on lymphoid tissue BECs in the mouse4-26. HVEs may use post-transcriptional mechanisms to regulate these inflammation- and lymphocyte migration–associated adhesion receptors.

In conclusion, through analyses of transcriptomes of lymphoid tissue capeillary and post-capillary high endothelium, we have defined genes and programs for EC specialization and for control of lymphocyte recruitment, and have identified previously unknown mechanisms involved. Beyond the analyses provided here, our data should provide a resource for the discovery of additional mechanisms of vascular specialization and function, and for the selection of markers and genes for targeted therapies or genetic manipulation.

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

Accession codes. GEO: microarray data, GSE8056.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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ONLINE METHODS

Reagents. Phycocyanin–indotricarbocyanine–conjugated anti-CD31 (390), peridinin chlorophyll protein–cyanine 5.5–conjugated anti-CD45 (30–F11), phycocyanin–conjugated anti-Gp38 (eBio8.1.1), allophycocyanin–conjugated anti-Ly-6C (H1K.4) and peridinin chlorophyll protein–cyanine 5.5–conjugated anti-TER-119 (TER-119) were from eBioscience. Phycocyanin–indotricarbocyanine–conjugated anti-CD14 (145–2C11), allophycocyanin–indotricarbocyanine–conjugated anti-CD19 (6D5), allophycocyanin–conjugated anti-IgD (11.26c.2a), peridinin chlorophyll protein–cyanine 5.5–conjugated anti-CD326 (G8.8), peridinin chlorophyll protein–cyanine 5.5–conjugated anti-CD11a (H155–78), allophycocyanin–conjugated anti-BST-1 (BP-3), phycocyanin–conjugated anti-CD63 (NVG-2) and anti-GFP (FM264G) were from Biolegend. Antibody to mouse PARM-1 (EPR10009) was from Abcam. DyLight 488–conjugated donkey antibody to rat IgG (712-486-153), DyLight 488–conjugated donkey antibody to goat IgG (705–486-147) and Alexa Fluor 488–conjugated donkey antibody to rabbit IgG (711–546–152) were from Jackson ImmunoResearch Laboratories. Antibodies S2, HECA-452, MECA-79, MECA-367 and MECA-99 were produced in-house from hybridomas and were labeled with fluorophores through the use of a DyLight Antibody Labeling Kit (Thermo Fisher Scientific). Phycocyanin–conjugated goat F(ab′)2 antibody to human IgG (H10104), CFSE (carboxyfluorescein succinimidyl ester) and CellTracker Violet (CTV) were from Invitrogen. Collagenase P and dispase II, neutral protease, grade II were from Roche. DNase I from bovine pancreas was from Sigma. Fluorescein isothiocyanate–labeled Sambucus Nigra (SNA) lectin was from Vector laboratories. Polyclonal goat antibody to mouse Nr1p (AF566) and mouse CD22–Fc fusion proteins were from R&D Systems. All reagents were ‘titered’ or used according to the manufacturers’ recommendations. Antibodies used for immunoprecipitation and immunoblot analysis are identified below.

Mice. 6- to 8-week-old male and female BALB/c mice were used for endothelial isolation for flow cytometry and cell sorting, and for tissue isolation for immunofluorescence. For some immunofluorescence staining, 6-month-old male and female Hes1-EmGFP mice55 (which have a transgene encoding GFP knocked into the gene encoding Hes1) were used. These mice were bred and maintained in the animal facilities of the Veterans Affairs Palo Alto Health Care System, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. For lymphocyte-trafficking studies, wild-type mice, Cdl22+ mice56 and S6gal1−/− mice57 (on the C57BL/6J background) were bred and housed at The Scripps Research Institute. All animals were maintained and bled in specific pathogen–free conditions in the animal facility of the Veterans Affairs Palo Alto Health Care System or Scripps Research Institute, and all animal work was approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Palo Alto Health Care System, or by relevant animal care committees at The Scripps Research Institute.

Preparation of lymphoid tissue ECs for flow cytometry. PLNs (inguinal, axillary and brachial lymph nodes) and MLNs and PPs were carefully isolated from equal numbers of male and female BALB/c mice. Tissues were lightly minced in ice-cold Hank’s balanced-salt solution (HBSS) and were collected by 2 min of centrifugation at 100 g at 4 °C. For preparation of PPs, additional wash steps (twelve washes in 5 ml ice-cold HBSS with transfer pipets) were performed before tissues were minced. Supernatants containing released lymphocytes, stromal cells, mucus and fat tissues were discarded, and pellets were then digested for 60 min at 37 °C with gentle rocking in HBSS (with calcium and magnesium) medium containing 0.2 mg/ml collagenase P, 0.8 mg/ml dispase II and 0.01 mg/ml DNase. Digestion was stopped by the addition of FBS (final concentration, 30%) on ice. Dissociated cell suspensions were then passed through 100-µm filter follow by passage through a 40-µm filter. HECs and CAP ECs were ‘enriched’ from the resulting cell suspensions by depletion of hematolymphoid cells with anti-CD45 mouse MicroBeads according to the manufacturer’s protocol (Miltenyi). For flow cytometry, the ‘enriched’ ECs were labeled by standard protocols with fluorochrome–conjugated antibodies (identified above) or with CD22–Fc protein in HBSS containing 2% FBS. Dead cells were excluded by staining with propidium iodide (Sigma). Background fluorescence was determined by the ‘fluorescence-minus–one’ method. Flow cytometry data were acquired on an LSR II or LSRFortessa (BD Biosciences) with FACSDiva software (BD Biosciences). FlowJo software (TreeStar) was used for further analysis.

Microscopy. PLNs (inguinal, axillary and brachial lymph nodes) and PPs isolated from male and female BALB/c mice were placed in Tissue-Tek compound (Sakura) and were frozen at −80 °C. Cryosections of tissues fixed in acetone or 4% paraformaldehyde were stained with antibodies (identified above) according to standard protocols. Staining was imaged on an LSM 710 confocal microscope (Carl Zeiss).

Immunoprecipitation. PLNs (inguinal, axillary, and brachial lymph nodes) and/or MLNs were isolated from male and female C57BL/6j mice, and membrane–enriched protein fractions were produced with a ProteoExtract Native Membrane Protein Extraction Kit according to the manufacturer’s instructions (Calbiochem). Rabbit monoclonal antibody to Parm1 (1:100 dilution; EPR10009; Abcam) and Protein G Dynabeads (Life Technologies) were used for immunoprecipitation of Parm1 from the membrane–enriched protein fraction. Monoclonal rabbit IgG antibody to Hsp90 (1:100 dilution; C45G5; Cell Signaling Technology) served as an immunoglobulin control for immunoprecipitation. Immunoprecipitates were denatured in SDS loading dye, were separated by 7.5% SDS-PAGE and were transferred to PVDF membranes. Immunoblots were probed with the primary antibodies anti-Parm1 (1:1,000 dilution; EPR10009; Abcam) and MECA-79 (2 µg/ml) produced in-house and the secondary antibodies IRDye 800CW–conjugated donkey anti-rabbit (1:5000 dilution; 926-32213; LI-COR) and Alexa Fluor 680–conjugated goat anti-Rat IgM (1/10000 dilution; 112-625-075; Jackson ImmunoResearch), respectively. Images were acquired with the LI-COR Odyssey CLX infrared imaging system.

Microarray analysis. Tissues were obtained from equal numbers of 6- to 8-week-old male and female BALB/c mice (typically 10–14 per experiment) and underwent enrichment for ECs by depletion with anti-CD45 as above. Tissue processing time (from the time the first mouse was killed to the time sorting began) was generally less than 4 h. The ‘enriched’ ECs were stained with antibodies (identified above) and propidium iodide for the exclusion of dead cells, and were sorted on a FACSAria III with a 100-µm nozzle (BD Biosciences), with a flow rate setting between 1 and 2 (fewer than 2,500 events per second). Monoclonal antibody to gp38 (podoplanin), monoclonal antibody to CD31 and monoclonal antibodies to lineage markers (CD45, CD11a, Ter-119 and EpCAM (CD326)) (all antibodies identified above) were used as described6 to define ECs (lineage marker–negative CD31+gp38+ blood ECs) and to separate ECs from other cell types (lymphatic ECs, fibroblastic reticular cells, hematolymphoid cells). Lineage markers for exclusion included CD14, the elimination of most hematolymphoid cells; Ter-119, for the elimination of erythrocytes; LFA-1, for the elimination of plasma cells; and EpCAM, for the elimination of epithelial cells. Gated ECs were further sorted with MECA-79 and/or MECA-367 to define HEVs and with MECA-99 to define capillary endothelium. In some experiments, MLN HEVs were separately sorted into MECA-79+MECA-367+ and MECA-79+MECA-367− subsets; these samples are included in the submitted microarray data, but because of the similarity of their overall gene expression, all MLN samples were pooled for the analyses presented here. Sorted cells were collected directly into RLT buffer (Qiagen). Sort purity, estimated by reanalysis of cells sorted under identical conditions, was at least 95% for all analyzed samples (Supplementary Fig. 3).

RNA was isolated from the sorted BEC subsets with an RNeasy Plus Micro kit (Qiagen). 5–20 ng of total RNA from each sample (with an RNA integrity number of at least 8, as determined by Agilent bioanalyzer at the Stanford University PAN facility) was used for amplification, labeling and hybridization, done by Expression Analysis. Samples were hybridized on Affymetrix GeneChip Mouse Gene 1.0 ST arrays. GeneSpring GX 12.6 and Partek Genomic Suite software (6.6) were used for processing and analysis of the data. GeneSpring preprocessing and default normalization (RMA-16) were applied.

Quality control determination was performed as described by the Immunological Genome Project (ImmGen) Consortium5. For all samples analyzed in this study, positive values versus negative values for the area under the curve were at least 0.8, and dynamic ranges were at least 40 (average of all 19 samples was 59 with a standard error of 1). Cellular contamination was
assessed by evaluation of transcripts from genes known to have high expression by potential contaminating cell populations, as follows: for T cells, Tcf7, Fyb, Lat, Thy1 and Cd3g; for B cells, Igk, Cd29h, Igk-6, Msa4a, Pax5, Igj and Igk; for epithelial cells, Krt19, Krt11, Epcam, Muc1 and Cd1h1; for myeloid cells, Anxa2, Alox15, Il13ra1, Tlr13 and Il13ra2; for platelets, Gp1ba, Iga2b, Mpl, and Gp9 and Epor; for red blood cells, Hba-a1 and Hba-a2; and for sign of cellular stress, Hspa8. By these criteria, contamination by known cell types was low. Cellular purity was similar to that of stromal cell samples of the ImmGen Consortium.

For the generation of gene-expression data sets comparing endothelial subsets and lymphocytes, mouse gene-expression data from the ImmGen Consortium were obtained from the GEO website (GSE15907).

In addition to staining capillary ECs, MECA-99 stained arterial ECs (M.D.L. and E.C.B., unpublished data). In sections of lymphoid tissues, the percentage of MECA-99+ endothelium that was within arteries was estimated to be ≤5%, as determined by morphometric analysis of multiple sections of PLNs and PPs with the aid of elastin-binding fluorescent dye to define arterial ECs and to distinguish them from capillary endothelium. We therefore refer to sorted addressin-negative MECA-99+ ECs as ‘CAP ECs’ throughout the manuscript.

PP CAP samples underwent additional analysis to evaluate the possibility of contamination from endothelium from non-PP small intestine. For this, PPs were clipped carefully from the small intestine, minimizing the inclusion of non-PP gut wall. Sections of multiple PP were analyzed morphometrically with an ocular grid to determine the proportion of lymphoid tissue versus nonlymphoid lamina propria and muscularis. The area of PPs was defined by immunofluorescence staining for B cells and T cells (anti-IgD; 11.26c.2a; BD Biosciences) and anti-Cd3 (145-2c11; eBioscience)). Morphometric analyses indicated that ~78% (± 1% (± s.e.m.), from the evaluation of at least six PPs per mouse from two mice in each) of the isolated tissues comprised PP lymphoid tissue. Moreover, the recovery of CAP ECs from PPs was much more efficient than from non-PP intestines with our isolation protocol. For this comparison, PP were removed from small intestine, and ECs were dissociated enzymatically from that small intestine by digestion of small intestine fragments of size similar to that of dissected PPs. MECA-367+/MECA-99– ECs were either rare or too weakly reactive with MECA-367 to allow detection among the resulting small intestine ECs (data not shown), which confirmed removal of PPs. Dissociated CD31+gp38+ ECs were counted by flow cytometry. Almost all were MECA-99+ . Twice as many BECs were recovered from PPs than from non-PP gut wall (per unit wet weight; two independent experiments with four mice in each). From these analyses, we estimated ~12% contamination of PP lymphoid tissue CAP with MECA-99+ CD31+ ECs from extralymphoid gut wall.

As in any whole-genome expression study of cells derived from in vivo sources, expression of individual genes in our data should be interpreted with caution, since the possibility of signals from contaminating cells cannot be formally excluded. However, in addition to ruling out substantial contamination of our sorted cells by other characterized lymphoid tissue cells types (by the evaluation of the cell-specific marker genes described above), we separately determined whether other data sources could provide independent confirmation of EC expression of the top five genes (‘test genes’) with the greatest difference in expression in HEV or CAP signature gene sets, and in PLN HEV– versus–PP HEV signature genes (i.e., Figs. 2b and 5a). Expression of most of the test genes has been observed in cultured BECs or confirmed by immunohistochemical staining of BECs in tissue sections, as reported in the literature. For other genes, we evaluated endothelial gene expression as reported in public data sets. We analyzed deposited data obtained from cultured human or mouse BECs and considered expression in the top 25% of genes as indicating meaningful expression in ECs. We also took advantage of data sets from the ImmGen Consortium to assess expression of the test genes in the following cell subsets: sorted mixed BECs from PLNs and MLNs in independent studies of C57BL/6 mice; B lymphocytes and T lymphocytes and dendritic cell subsets; lymphatic ECs; fibroblastic reticular cells; and CD31− gp38− (‘double-negative’) stromal cell samples enriched for pericytes. Together, the stromal cells analyzed by the ImmGen Consortium encompass all dissociated stromal (CD45+) cells released enzymatically. Most ‘test’ genes had high expression by total BECs in the database of the ImmGen Consortium, and many genes had higher expression in BECs than in any other stromal subset (defined by the ImmGen Consortium) in PLNs or MLNs, or in lymphocytes, dendritic cells or macrophages. Endothelial expression of all ‘top five’ signature genes was supported by one or more of these criteria. Together, these considerations suggest that genes with the highest expression and greatest difference in expression in our analyses are expressed by the target EC subsets themselves.

However, four genes expressed by cultured ECs that had high expression in our samples had only weak expression or no expression in the lymph node BECs analyzed by the ImmGen Consortium, even though these BECs should comprise a mixture of CAP and HECs. Tcf21, Tshr, Pfa4 and Fjx1, which had high expression in the HEVs we sorted from male and female BALB/c mice, had only weak expression or no expression (EV < 120) in the analysis by the ImmGen Consortium of lymph node BECs, which were sorted from male C57BL/6 mice. Since each of these genes has been observed to be expressed by cultured BECs or by endothelium in vivo in other settings, these results suggest strain-specific differences in expression of genes by BECs, although sex-specific differences are also possible.

Short-term homing assays. Donor splenocytes were isolated from wild-type or Cd22−/− mice and were labeled with CTv or CFSE. Labeling of the CD31+ gp38+ cells was determined by CtV and CFSE, respectively. The Cd22−/− cells were labeled with CFSE, and in two other, the labels were reversed. Under these conditions, the in vivo homing activity of cells was independent of the label. 60 × 10^6 labeled cells (30 × 10^6 from wild-type mice and 30 × 10^6 from Cd22−/− mice) were transferred into wild-type or St6gal1−/− recipient mice by injection into the tail vein. After 1.5 h, lymphocytes from the PLNs (inguinal, axillary and brachial lymph nodes), MLNs and PPs of recipient mice were isolated, stained with anti-Cd3, anti-Cd19 and anti-lqD (identified above) to define T cell and B cell subsets, and cells were analyzed by flow cytometry. For each experiment, the homing of IgD+ B cells and CD3+ T cells from wild-type and Cd22−/− donors was evaluated. Results are presented as the relative localization ratio (RLR), which is calculated by normalizing the efficiency of homing of each subset to that of wild-type CD3+ T cells in each organ.

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\text{RLR(organ of population 'a' in organ Z) = \frac{\text{`a' donor cells in organ Z / `a' cells injected}}{\text{CD3 + WT donor cells in organ Z / CD3 + WT cells injected}}}
\]

Statistical analysis. The statistical significance of differences between sets of data was assessed by a two-tailed unpaired Student's t-test unless stated otherwise. Error bars shown indicate standard errors unless otherwise indicated. Analytic methods for the significance of differences in gene expression are indicated in the text. Significance of clusters was determined by multiple bootstrap resampling.

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Corrigendum: Transcriptional programs of the lymphoid tissue capillary and high endothelium reveal control mechanisms for lymphocyte homing

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In the version of this article initially published, Hiroto Kawashima was omitted as an author. The correct author list is as follows: Mike Lee¹, Helena Kiefel¹, Melissa D LaJevic¹, Matthew S Macauley², Hiroto Kawashima³, Edward O’Hara⁴, Junliang Pan⁴, James C Paulson² & Eugene C Butcher¹,4,5.

The affiliation for this author is as follows: Department of Biochemistry, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, Tokyo, Japan. The Author Contributions section should include “H.K. provided advice and the S2 hybridoma” (and the corresponding first thanks in Acknowledgments should be removed). The error has been corrected in the HTML and PDF versions of the article.