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

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Abstract

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 displayed gene programs for vascular development. HEVs were enriched in genes for immune defense and lymphocyte migration. We identify capillary and HEV markers and candidate mechanisms for regulated lymphocyte recruitment 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.

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CONTRIBUTIONS

M.L. developed HEC isolation protocols, designed and performed experiments including EC flow cytometry, whole genome expression analyses, immunohistology, and homing studies. H. Kiefel performed flow cytometry and immunofluorescence staining. M.D.L. performed immunoprecipitation and immunoblotting studies. M.S.M. did homing studies. M.S.M. and J.C.P. contributed to writing of the section on HEV glycans. H. Kawashima provided critical reagents and advice. E.O. established the parameters for HEC RNA isolation. J.P. provided intellectual input. E.C.B. designed and guided the study, analyzed data and wrote the manuscript.

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Introduction

The blood vascular endothelium in lymphoid tissues controls homeostatic lymphocyte homing and leukocyte recruitment during inflammation, regulates metabolite exchange and blood flow to meet the energy requirements of the immune response, and maintains vascular integrity and hemostasis. These diverse functions require specialization of the endothelium. In lymphoid tissues, the capillary network is thought to be primarily responsible for solute and fluid exchange whereas post-capillary high endothelial venules (HEVs) are specialized for lymphocyte recruitment¹⁻³. In addition, HEVs display tissue specialization. HEVs of skin-draining peripheral lymph nodes (PLN) and the gut-associated lymphoid tissues (GALT; including Peyer's patches (PPs) and mesenteric lymph nodes (MLNs)) express tissue specific vascular “addressins”, adhesion receptors that together with chemokines control the specificity of lymphocyte homing⁴. In spite of the importance of vascular specialization to the function of the immune system, little is known about the transcriptional programs that define HEV specialization³. Recent studies have demonstrated the feasibility of isolating mouse lymphoid tissue endothelial cells for transcriptional profiling and have characterized unique transcriptomes of blood versus lymphatic endothelial cells⁵.

Here we describe transcriptional programs of high endothelial cells (HECs) and capillary endothelia (CAP) from PLN, MLNs and the gut-associated PPs. This study defines transcriptional networks that discriminate capillary 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 HECs, including mechanisms for B cell recruitment 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 lymphocyte homing into lymphoid tissues, and to the regulation of local immune responses.

Results

Transcriptional specialization of lymph node and PP BEC

We generated whole-genome expression profiles of lymphoid tissue blood vascular endothelial cell (BEC) subsets using minor modifications of established protocols⁵. As illustrated in **Fig. 1a**, HEC were sorted from PLN BEC using monoclonal antibody (MAb) MECA-79 to the peripheral node addressin (PNAd), which comprises sulfated carbohydrate ligands for the lymphocyte homing receptor L-selectin (CD62L). PP HECs were defined by MAb MECA-367 to the mucosal vascular addressin MAdCAM1, an (Ig) family ligand for the gut lymphocyte homing receptor $\alpha_4\beta_7$. CAP were defined by reactivity with MECA-99, an EC-specific antibody⁶ of unknown antigen specificity that distinguishes lymphoid tissue CAP from HEVs (**Fig. 1b** and see **Supplementary Methods**).

To identify sources of variability in gene expression, we applied principal component analysis (PCA) to profiles of genes selected for different expression (2-fold difference, $P < 0.05$ by one-way ANOVA between any pair of samples) and for raw expression value (EV) >140 . Biological replicates clustered together, indicating low biological and inter-procedural

variation (**Fig. 1c**). The first principal component (the largest difference between samples) separates CAP from HECs, emphasizing conserved patterns of segmental gene expression by CAP versus HEVs. Tissue-specific differences in gene expression dominate the second principal component. While specialization of lymph node versus gut-associated HEVs is well described in terms of vascular addressins, the PCA analysis revealed robust tissue specific differences in CAP transcriptomes as well. This suggests a previously unappreciated specialization of the PP versus PLN capillary vasculature. MLNs are known to share features of both PLNs (for example, expression of PNAd by most HEVs), as well as characteristics of PP (expression of MAdCAM1 by subsets of MLN HEVs). Consistent with this, the transcriptional profiles of MLN HECs fall between those of their PLN and PP counterparts. Clustering using Pearson's correlation confirms the significance of sample clusters that reflect tissue and segmental differences in gene expression (**Fig. 1d**).

HEV vs. CAP gene expression signatures and pathways

To define HEV and CAP specific transcriptional signatures, we compared HECs versus CAP from PLNs, MLN, and PPs. Within each tissue, we identified genes expressed (EV >140) by CAP or HECs, and differing at least 1.5 fold between HEC and CAP (gene counts shown in **Fig. 2a**). Genes whose expression was elevated in CAP or in HECs in all three tissues were used for gene ontology (GO) term and pathway analyses (see below). These HEC (799 genes) and CAP (642 genes) signature gene sets are listed in **Supplementary Table 1**. We also identified 100 highly expressed genes that differ by at least 4-fold between HECs versus CAP, EV>900 (**Fig. 2b**).

We initially sought additional cell surface markers of lymphoid tissue endothelial specialization, both to validate the identity of the sorted cells and to assess potential heterogeneity. We identified CD63, a tetraspanin protein implicated in P-selectin function on activated EC⁷, as an HEV marker that uniformly and selectively decorated dissociated HECs, but was weak or absent on CAP, correlating with gene expression (**Fig. 2c**). Capillaries uniformly expressed Ly6C, as assessed by flow cytometry, whereas HEVs were poorly stained correlating with gene expression (**Fig. 2d**). We previously identified Ly6C as a microvessel antigen in lymph nodes⁸. The unimodal expression of Ly6C and MECA-99 antigen by dissociated CD31⁺ addressin-negative BECs suggests that sorted CAP comprise a relatively homogeneous EC population.

As expected given the morphology and histochemical properties of HEVs, gene ontology analyses of HEC signature genes revealed enrichment for genes 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 GO terms for defense, inflammatory response, chemokine activity and lymph node development, as well as genes in the NF- κ B signaling pathway. HEVs play key roles in the development of lymphoid tissues including lymph nodes and PPs in perinatal life, but also tertiary lymphoid tissues in sites of chronic inflammation. NF- κ B signaling through lymphotoxin is required for maintenance of HEVs *in vivo*³, and tumor necrosis factor (TNF) and Toll-like receptor ligands signal through NF- κ B to induce vascular adhesion receptors and chemoattractants for leukocyte recruitment. Pathway

analyses (KEGG and Enrichr) confirmed enrichment for genes involved in glycan synthesis and metabolism, and in sphingolipid metabolism (not shown). As expected, HECs expressed the master venous regulator *Nr2f2* (*COUP-TFII*; **Fig. 3b** bottom). The analysis did not reveal HEV enrichment for cardiovascular or endothelial-specific GO terms.

In contrast, GO terms related to endothelial development and angiogenesis featured prominently among CAP signature genes (**Fig 3a**). CAP were also enriched in genes for pathways involved in vascular differentiation, including Wnt, transforming growth factor- β (TGF- β) and Notch signaling.

Interestingly, CAP expressed genes associated with arterial specification during embryonic vasculogenesis, including *Notch4*, *Efnb2*, *Nrp1*, *Jag2*, *Dll4*, *Gja5*, *Hes1*, and *Kdr* (**Fig. 3b**)^{9, 10}. Immunofluorescence staining confirmed expression of *Nrp1* (**Fig. 3c**) and *Hes1* (**Fig. 3d** and **Supplementary Fig. 1**) by MECA-99⁺ capillaries. In contrast, HECs expressed the master venous regulator *Nr2f2* (*COUP-TFII*; **Fig. 3b** bottom). As suggested by GO analysis, CAP also highly and selectively expressed several genes implicated in angiogenesis, including *Esm1*, *Bgn* (*Biglycan*), and several angiogenesis-associated G protein-coupled receptors (GPCRs) and their ligands, such as *Cxcl12* and *Cxcr4*. *Esm1* is involved in angiogenic sprouting, but is also a secreted ligand for LFA-1 and inhibitor of leukocyte β_2 integrin-mediated leukocyte adhesion¹¹; it may help prevent leukocyte arrest in capillaries. CAP also expressed multiple growth factors and receptors (**Fig. 3b**). Genes for all three VEGF receptors (*Flt1*, *Flt4* and *Kdr*) and for *Vegfc* were preferentially expressed by CAP, whereas *Vegfb* is higher in HEC and *Vegfa* is expressed by both HEV and CAP. Aquaporins 1, 7 and 11, which regulate tissue fluid, glycerol and potentially CO₂ exchange¹², were expressed exclusively (*Aqp7* and *11*) or more highly by CAP (**Fig. 2b**, and **Supplementary Table 1**). The results reveal transcriptional control of anti-adhesive, angiogenic, and transport properties of the capillary endothelium.

HEC signature genes included several genes encoding proteins involved in innate defense, including components of the complement cascade (*C1s*, *Cfb*, decay-accelerating factor *Cd55*; **Fig. 3b**); *Pglyrp1*, a pattern receptor for peptidoglycans of Gram-positive bacteria; and the hepcidin antimicrobial protein *Hamp*. HECs also preferentially expressed genes for Serpins a3n and a1c, inhibitors of neutrophil proteases cathepsin G and elastase (The UniProt Consortium; <http://www.uniprot.org/>). Neutrophils roll on HECs and are activated during extravasation when lymph nodes are inflamed; the presence of these inhibitors may prevent EC damage. Although genes involved in angiogenesis tend to be enriched in CAP, HEVs more highly expressed LRG1, an HEV marker and regulator of EC TGF- β signaling implicated in neovascularization¹³. *Lyve1*, a marker of lymphatic EC, was expressed by HEC more highly than by CAP (but much less than by lymphatic EC⁵). HEV signature genes involved in NF- κ B signaling include ubiquitin D, which facilitates degradation of inhibitory I κ B (**Supplementary Table 1**), and the EC-specific TNF family member *Tnfsf15* which activates NF- κ B and serves as an autocrine inhibitor of endothelial growth and modulator of vascular homeostasis (The UniProt Consortium).

Chemokines, cytokines, their receptors, and GPCRs

HEVs as well as CAP expressed genes for receptors for immune cytokines (**Fig. 4a**). Genes encoding the IL1 receptor *IL1r1* and several TNF receptor family members (*Tnfrsf9*, *Tnfrsf11a*, *Relt*, and *Eda2r*) were preferentially expressed in HEVs, while *Fas* and *Tnfrsf11b* were higher in CAP. *Tnfrsf1a* and *Ltbr* were uniformly high in both HEVs and CAP. *IL3ra*, *Csf2ra* and their common beta chain co-receptor *Csf2rb* were expressed by CAP and HEVs. *IL2rg*, the common gamma chain, was highly expressed and somewhat preferentially by HEVs. While HEVs and CAP similarly expressed genes for type 1 interferon (IFN) and IFN- γ receptors, HEVs expressed *Ifngr2* more highly than CAP. Transcripts for receptors for IL-27, IL-11, oncostatin M, and leukocyte inhibitory factor (*IL27ra*, *Osmr*, *Il11ra* and *Lifr*) and their common partner chain *Il6st* (gp130) were expressed by HEVs; expression of *IL27ra* and *Il6st* was HEV selective. Interestingly, CAP but not HECs constitutively expressed transcript for IL-6, which is cytoprotective for ECs¹⁴, whereas *Il6ra* was expressed in both HEV and CAP. Thus HEVs and CAP have both distinct and overlapping receptors for homeostatic and inflammatory cytokines.

In the multi-step 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 HEC, or can be delivered to EC from surrounding tissues or lymph; they can be presented on the luminal surface of EC by binding to heparan sulfate proteoglycans (HSPGs), glycosaminoglycans that also bind growth and other factors (reviewed¹⁵). We identified chemokines and receptors expressed in HEVs or CAP (EV >140) (**Fig. 4a**). Gene expression confirms high-level HEC-specific expression of transcripts for CCL21, which triggers lymphocyte arrest on HEVs^{1, 4, 16}. Surprisingly, HECs also constitutively expressed genes for CXCL10 and CXCL11: these chemokines function as ligands for the inflammatory trafficking receptor CXCR3^{17, 18}. Although CXCL12 and CXCL13 are displayed by HEVs and participate in B cell recruitment in PPs¹⁷, HEV expressed little transcript for these chemokines which therefore likely derive from surrounding stromal sources. Such tissue-derived chemokines, as well as chemokines arriving in lymph, can be transported from the abluminal to luminal surface of venular EC by *Ackr1* (*Darc*), a unique non-signaling chemokine receptor specialized for this function¹⁸. *Ackr1* is expressed highly by HEV but not CAP in our samples. HEVs also expressed *Ackr2* (*Ccbp2*), which encodes the scavenger receptor Ackr2(also known as D6) that functions to internalize and clear chemokines from the cell surface¹⁸. Genes for several HSPG core proteins were differently expressed by HEVs and CAP as well (**Fig. 4a**). Differential expression of these proteins, as well as EC-subset-selective modifications of their heparan sulfate side chains¹⁵, could regulate chemokine display. Together the results demonstrate transcriptional control not only of EC chemokine expression, but also of endothelial mechanisms of chemokine transport, presentation and degradation.

Chemokines and other GPCR ligands also regulate endothelial responses¹⁹. Transcripts for CXCL12 and its receptor CXCR4 were selectively expressed by CAP, where they may regulate endothelial migration and angiogenesis. Interestingly, CAP also constitutively expressed CX3CL1, which encodes the transmembrane chemokine fractalkine. Fractalkine is constitutively expressed by arterial endothelium, is reportedly induced in capillary and

arterial but not venous endothelium *in vivo* by TNF²⁰, and can mediate angiogenesis²¹. The long amino terminal GPCR, CD97, which may regulate adherens junction strengthening and induce angiogenesis, was selectively expressed by CAP, as was the endothelin receptor Ednrb. Ednrb is involved in generation of nitric oxide, promoting microcirculation. The CXCR3 ligands CXCL10 and CXCL11, transcriptionally expressed by HEC, are angiostatic^{17, 18}.

Together the results show that CAP and HEVs differentially express an array of ligands and receptors that can mediate communication with the local environment to control leukocyte recruitment and regulate segmental endothelial cell responses.

Ig family, mucin and enzyme receptors for lymphocyte 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. *Cd34* was highly expressed in both capillaries and HEV, whereas *Glycam1* was preferentially expressed in HEVs. Podocalyxin-like (*Podxl*) can accept L-selectin binding glycotopes and is reportedly expressed by HEVs²², but our data reveal preferential *Podxl* expression in CAP (**Fig. 4b**), suggesting that its role in cell repulsion and EC tube formation²³ may be more important. CD300lg (Nepmucin), which presents L-selectin ligands and also binds lymphocytes by its N terminal V-type Ig domain, is displayed by PLN but not PP HEVs²⁴, correlating with its differential expression on HEV shown here. However *CD300lg* and *Ecmn*, which had a similar expression pattern, are both somewhat more highly expressed by CAP than HEV. Our gene profiling also revealed selective HEV expression of *Parm1*²⁵ encoding the prostate androgen regulated mucin 1 (Parm1). Immunofluorescence histology confirmed expression of Parm1 (**Fig. 4c**), a mucin not previously described on HEVs, and immunoblot analysis demonstrated decoration of Parm1 by PNAd glycotopes as indicated by MECA-79 reactivity (**Supplementary Fig. 2**).

Transcripts for the β_2 integrin ligands ICAM1, which mediates arrest of rolling lymphocytes on HEV, and ICAM2 were expressed by lymphoid HEVs and CAP. The $\alpha_4\beta_1$ integrin ligand VCAM1 was highly expressed (EV ~1000) in all lymphoid EC subsets, as well, even though this vascular adhesion molecule is not detectably expressed at the protein level by ECs in LNs or PPs. Similarly vascular E and P selectin, although difficult to detect on resting HEVs, were well represented in HECs at the RNA level. Although we cannot exclude upregulation of genes during EC isolation, the results suggest that expression of VCAM1 and the vascular selectins may be regulated post-transcriptionally in BECs *in vivo*.

Among other genes implicated in lymphocyte homing via HEV, *Stab1* (encoding common lymphatic endothelial and vascular receptor CLEVER1)²⁶ was uniformly expressed by CAP and HEVs (**Fig. 4b**). *Aoc3* encoding inducible vascular adhesion protein 1 (VAP1)²⁷ was highly expressed by CAP but not HEC in our samples (**Fig. 4b**); although VAP1 constitutively decorates HECs in humans²⁷ (and M.D.L. and E.C.B., personal observations), lack of *Aoc3* expression in HECs in our samples suggest that HEV-associated VAP1 immunostaining observed in resting mouse LNs may be on pericytes.

Genes for lipid mediators of lymphocyte migration

HEVs expressed genes involved in the synthesis and transport of lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), lipid mediators of lymphocyte motility and chemotaxis. HEVs as well as CAP expressed *Enpp2* encoding autotaxin, which is functionally important for LPA generation and lymphocyte recruitment via HEVs^{24, 28}. *Sphk1* and *Asah2*, encoding sphingosine kinase and acylsphingosine deacylase 2 involved in S1P synthesis, were preferentially expressed by HEV (**Fig. 4b**). *Asah2* generates sphingosine from N-acylsphingosine, and *Sphk1* phosphorylates sphingosine to S1P. S1P potently stimulates lymphocyte motility, and through the T cell S1P receptor 1 (*S1pr1*) enhances T cell integrin-dependent arrest in PLN but not PP²⁹. This tissue difference in S1P activation of T cell arrest may relate to higher *Sphk1* expression observed in PLN than PP HEVs (1.5 fold higher in PLN vs PP HEC, $P < 0.05$). *Sphk1* is an intracellular enzyme, but HEV and CAP also expressed *Spps2* encoding the S1P transporter (**Fig. 4b**) which is required for S1P support of lymphocyte exit from bone marrow and thymus. Autocrine production or exogenous sources of S1P and LPA likely affect ECs directly, as well, since BECs highly expressed *S1pr1* and both *Lpar4* and 6. *Lpar6* (*P2y5*) is preferentially expressed by CAP.

HEVs but not CAP highly expressed *Ch25h* encoding Cholesterol 25-hydroxylase, which synthesizes 25-hydroxycholesterol (25-OHC). PPs and to a lesser extent PLN HEVs also expressed *CYP27a1* which generates 27-hydroxycholesterol (27-OHC) (**Fig. 4b**). These sterols are the immediate precursors of potent chemoattractant ligands for the lymphocyte receptor Gpr183 (also known as EBI2)^{30, 31}. However, HEV also expressed transcripts for hydroxysteroid dehydrogenase HSD3B7, which degrades Gpr183 ligands (**Fig. 4b**); but lack the enzyme CYP7B1 required for their generation.

Differently expressed transcription factors

BEC subsets in lymphoid tissues differently express transcripts for an array of transcription factors (TFs, **Fig. 4a**) including ligand-activated TFs (e.g. *Ar* encoding the androgen receptor, expressed by HECs, and *Pparg* and the retinoic acid receptor *Rarg* expressed more highly by CAP); TFs implicated in cardiovascular development (e.g. *Sox17*, *Msx1*, *Id1* and *Id3*, *Junb*, *Meox2*); and TFs involved in regionalization or digestive system development (e.g., *FoxP4*, *Hlx*, *Hoxd8*, *Lhx2*, *Egr2*, *TCF7l1*, *Meis2*). Notably, PP (but not PLN) HEC and CAP both express *NKX2-3*. *NKX2-3* is a homeobox TF involved in GI tract development that is required for EC MAdCAM1 expression *in vivo*³². These genes may help control the segmental and tissue specialization of GALT versus PLN HEVs.

Tissue-specific specialization of HECs

To assess tissue specific specialization of HECs we focused on genes differently expressed by PLN versus PP HEVs. PLN or PP HEV signature genes were defined to include genes expressed higher (>1.5 fold differ, $P < 0.05$) in PLN in comparison to PP HECs (or *vice versa*), to all lymphoid tissues CAP, and to naive and memory T cells (see **Supplementary Methods**). The resulting 150 PLN HEV signature genes and 48 PP HEV signature genes

were used for GO term analyses (**Supplementary Table 2**). We also identified the subset of these genes differing at least 2-fold between PP and PLN HEV (**Fig. 5a**).

As expected, key genes for PNA^d generation, *Fut7* and especially *Chst4*, were higher in PLN HECs while MAdCAM1 was high in PP HECs. *Bst1*, encoding a myeloid and EC surface ADP-ribosyl cyclase family receptor which has been implicated in neutrophil diapedesis³³, was preferentially expressed by HEC, and most highly in PLN HEC. Flow cytometric analysis confirmed both tissue (PLN versus PP) and segmental (HEV>CAP) differences in *Bst1* expression (**Fig. 5b**), correlating with gene expression. *Bst1* may have a role in tissue specific leukocyte recruitment via HEV.

GO analysis (selected list shown in **Fig. 5c**) revealed enrichment of PLN HEV signature genes for genesets for antigen processing and presentation, reflecting higher expression of MHC class II genes and the invariant chain CD74. PLN HECs were also enriched in genes for monocarboxylic acid biosynthesis, including *Sphk1* discussed above, and genes involved in prostaglandin D2 synthesis. Prostaglandin D2 is a selective attractant for CRTH2-expressing T cells (especially type 2 helper T cells). Interestingly, compared to PP, HEV in PLN expressed more *Ptgs1* encoding the constitutive cyclooxygenase 1 (Cox1; **Fig. 5a**), while inducible *Ptgs2* (Cox2) was expressed by both HEV nearly equivalently (**Fig. 2b**). PLN HEV also preferentially expressed ecto-5'-nucleotidase, *Nt5e* (CD73; **Fig. 4b**), encoding the rate-limiting enzyme involved in conversion of extracellular pro-inflammatory ADP and ATP into adenosine. Endothelial CD73 through adenosine generation and signaling has anti-inflammatory and tissue protective roles and regulates lymphocyte recruitment via HEV and leukocyte recruitment in models of inflammation²⁷.

GO analysis of PP HEV signature genes revealed enrichment in transcripts involved in “defense response” and “inflammatory response”, including genes *HDAC9*, and genes for chemokines CXCL10 and 11 which are classically upregulated in inflammation and recruit activated subsets of T cells as well as monocytes. Although their gene expression by HEV has not been reported, CXCL10 decorates HEV leading to the suggestion that HEV CXCL10 is derived from lymph or stromal cells¹³; our results suggest that, especially in PP, it may be endogenously expressed by HEC as well. *HDAC9* mediates pro-inflammatory epigenetic changes in immune cells, and also regulates angiogenesis. Interestingly, PP HEV-selective genes associated with “defense response” also include *Scd1*, which encodes a fatty acid desaturase that is induced by stress and maintains EC function³⁴. PLN and PP HEV also differ in genes involved in biosynthesis of sterols and lipids including prostaglandins. Prostaglandin transporter *Slco2b1* is in both PLN and PP HEV, but *Slco2a1* is highest in PP HEV and gut CAP, consistent with regional differences in eicosanoid biology. PP but not PLN HEV also expressed *Hsd11b2* (Corticosteroid 11-beta-dehydrogenase isozyme 2) which reduces intracellular cortisol, converting it to the inactive metabolite cortisone. GPR126, an adhesion GPCR, was exclusively expressed in PP and one MLN HEC preparation. Although GPR126 has not been detected previously in EC *in vivo*, perhaps due to its highly restricted expression, it is implicated in cardiovascular development and is upregulated by lipopolysaccharide in human umbilical vein endothelial cells³⁵. Together, these results suggest that gene expression in PP HEV reflects in part the greater steady state inflammatory and immune stimulation in PP compared to resting PLN.

Transcriptional control of L-selectin binding glycotopes

Glycoproteins of the endothelial surface undergo carbohydrate modifications that control lymphocyte adhesion (reviewed³⁶), as well as interactions of EC with growth factors and cytokines. We assessed the expression of genes involved in glycoconjugate formation (GO terms 0016757/0016932, supplemented by genes previously implicated in synthesis of HEV glycotopes). 215 of these genes were expressed (EV >140) in PLN and/or PP HEVs, including genes encoding each of the enzymes known to be involved in synthesis of the high affinity L-selectin ligand 6-sulfo-Sialyl LewisX (6-sulfo-SLeX)(**Fig. 6a**). Genes encoding enzymes responsible for synthesis of core 1 and branching core 2 N-acetylglucosamines (NAcLac), which comprise the framework for SLeX, were expressed equally by PLN and PP HEVs (**Fig. 6b**). These include genes for polypeptide GalNAc transferase 1 (*Galnt1*), Core 1 β 1-3 galactosyltransferase 1 (*C1galt1*), Core 2 branching GlcNAc transferase (*Gcnt1*), Core 1 extending β 1,3-N-acetylglucosaminyltransferase (*B3gnt3*), and members of the β -GlcNAc β 1,4-galactosyltransferase (B4GALT) family, *B4galt1* and *B4galt3-7*. B4GALT's responsible for NAcLac synthesis on HEV remain to be identified³⁶. *B4galt5* and 6 were preferentially expressed in HEVs, and thus are good candidates for participation in functional ligand synthesis.

In contrast to enzymes involved in NAcLac synthesis, genes for most enzymes responsible for terminal modifications required for L-selectin binding were expressed significantly higher in PLN than PP HEVs (at least 1.5 fold, $P < 0.05$; **Fig. 6b**). These include *Chst2* and *Chst4* that encode HEV carbohydrate (N-acetylglucosamine-6-O) sulfotransferases^{13, 37}. *Chst4* was expressed over ten-fold higher in PLN HEVs than in PP HEVs. *Chst2* was expressed highly by all HEVs, but displayed significant selectivity for PLN as well. *Chst4*^{-/-} mice have a more severe defect in lymphocyte homing to PLN than *Chst2*^{-/-} mice, and *Chst2/4* double-deficient mice display only minimal residual L-selectin-dependent lymphocyte rolling in PLN HEVs^{36, 37}. As reported, *Chst1* was also expressed by PLN and PP HEVs (but poorly if at all by CAP): it encodes keratan sulfate Gal-6 sulfotransferase which generates 6-sulfo-SLeX in culture models but does not contribute detectably to L-selectin mediated homing²². Genes for enzymes implicated in addition of terminal sialic acid and fucose residues of SLeX, *St3gal4* and *Fut7* respectively, were also significantly enriched in PLN HEVs ($P < 0.05$), although the difference in expression was small compared to that of *Chst4* (**Fig. 6b**). *St3gal4*^{-/-} mice have deficient L-selectin rolling in inflamed extralymphoid venules, but normal lymphocyte interactions with HEV³⁶. However, HEV expressed genes for each of the other known β -galactoside α 2,3-sialyltransferases as well, *St3gal1-3*, 5 and 6. *St3gal6* was particularly highly expressed by HEVs, although equally in PLNs and PPs. *Cmah* encoding cytidine monophosphate-N-acetylneuraminic acid hydroxylase, an enzyme that converts the terminal sialic acid of L-selectin ligands to N-glycolylneuraminic acid (Neu5Gc)³⁸, was highly expressed by HEVs, 1.7 fold higher in PLNs than PPs. Genes encoding HEV UDP-fucose and sulfate transporters, *Slc35c1* and *Slc26a2*, the latter reported in human tonsil HEVs³⁹, were also expressed slightly more highly by PLN HEVs. HEVs actively take up sulfate from the environment⁴⁰, and may import UDP-fucose as well to enhance substrates for 6-sulfo-SLeX synthesis. Overall, the data suggest that genes encoding key enzymes involved in the

terminal steps of L-selectin ligand synthesis are regulated in a tissue selective fashion on HEV, as are transporters that provide UDP-fucose and sulfate as enzyme substrates.

CAP show reduced expression of each of the regulated L-selectin ligand-encoding genes that distinguish PLN from PP HEVs (**Fig. 6b**). However, CAP were also deficient in the core 2 branching GlcNAc transferase *Gcnt1* (**Fig. 6a**). Branching core1 or core 2 glycans strengthen L-selectin mediated rolling through enhanced valency³⁶. Reduced core 2 branching may limit the potential for aberrant lymphocyte interactions in capillaries. CAP also expressed genes for glycosyltransferases that directly inhibit SLeX synthesis including *St3gal1*, which was higher in CAP than HEVs in both PLNs and PPs (**Fig. 6b**). *St3gal1* caps the proximal Gal β 1,3GalNAc of growing core 1 O-glycans, thus preventing the synthesis of core 1 or core 2 selectin ligands. Indeed deficiency of this enzyme leads to enhanced L-selectin ligand production by ECs and enhanced lymphocyte adhesion³⁶. CAP also expressed genes encoding α 2,8-sialyltransferases, including *St8sia4* that modifies N-glycans with anti-adhesive sialic acid polymers in the nervous system⁴¹. Together the results suggest that transcriptional programs play an important role in the segmental as well as tissue selective adhesive properties of EC glycoconjugates.

To correlate transcriptional profiles with cell surface expression, we used antibodies to relevant glycotopes (**Fig. 6c**)^{37, 42, 43}. HECA-452 recognizes sialic acid and fucose-dependent but sulfate independent SLeX- related epitopes⁴³. MECA-79 recognizes peripheral addressin 6-sulfo-SLeX on core1 but not core 2 O-glycans; recognition is sulfate but not sialic acid dependent³⁷. S2 recognizes 6-sulfo-SLeX and 6-sulfo-LacNAc on O- and N-glycans⁴². S2 stained dissociated PLN HECs much brighter (~ten-fold by flow cytometry) than PP HECs, although both were positive (**Fig. 6c,d**). MECA-79 stained PLN HEVs, but the surface of PP HEC was essentially negative. Immunohistochemical studies show abluminal but not luminal staining of PP HEVs with MECA-79¹. Our data raise the possibility that this abluminal MECA-79 reactivity derives from pericytes rather than HEC themselves, and indicate that most PP HEV 6-sulfo-SLeX glycotopes are on core 2 or N-glycans. Consistent with predictions from gene expression, the sulfate-independent SLeX epitopes recognized by HECA-452 decorated HEV in both tissues, and were only 2-3 fold more abundant on PLN than PP HECs. CAP stained poorly with all three mAbs (data not shown). The correlation of carbohydrate epitopes with patterns of glycosyltransferase and sulfotransferase gene expression suggests that transcriptional control mechanisms specify the segmental (capillary versus HEV) expression and tissue-specific specialization of modified glycans controlling L-selectin interactions.

***St6gal1* expression controls B cell homing to PP**

In contrast to genes responsible for L-selectin ligand synthesis, *St6gal1* was preferentially expressed by PP HEVs (**Fig. 6b**, top, and **Fig. 7b** left). It is moderately expressed by MLN HEV, but poorly by PLN HEV and by CAP in all tissues. ST6GAL1 is the sole enzyme outside the nervous system that adds sialic acid in α 2,6 linkage in the sequence Sia α 2-6Gal β 1-4GlcNAc (6'-sialyl-LacNAc; **Fig. 7a**) to terminate N- and O-linked glycan cores⁴⁴. This terminal modification has not been reported on LeX, and is believed to be mutually exclusive with the fucosylation required for generation of functional SLeX⁴⁵; thus

it may contribute to reduced L-selectin binding in PPs. 6'-sialyl-LacNAc is recognized by the *Sambucus nigra* (SNA) lectin, and flow cytometric staining with SNA confirms selective display of α 2,6-linked sialic acid by PP HEVs (**Fig. 7a**, right). Moreover, ST6GAL1 generates functional ligands for the B cell lectin CD22 (Siglec2)^{38, 46}, which in the mouse binds 6'-Sialyl-LacNAc with NeuGc as the sialic acid as a preferred ligand (e.g. NeuGc α 2-6Gal β 1-4GlcNAc)^{38, 47}. Conversion of CMP-NeuAc to CMP-NeuGc is carried out by cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH), and *Cmah* was highly expressed by HEVs (**Fig. 6b**)⁴⁷. Humans lack CMAH and human CD22 binds 6-sulfo-6'-sialyl-LacNAc (NeuAc α 2-6Gal β 1-4[6S]GlcNAc) as a preferred ligand⁴³. Expression of *St6gal1* in combination with *Cmah* and *Chst2* thus suggested that, among BEC subsets, PP HEV might uniquely display high-affinity CD22-binding glycans, and indeed a CD22-Ig chimeric protein robustly stained isolated PP HECs but not PLN HECs or CAP (**Fig. 7a**, right).

B cells home well to PPs but poorly to PLNs compared to T cells⁴⁸, but the mechanisms involved are poorly understood. To assess the role of CD22 and ST6GAL1 in B cell recruitment, we used short-term homing assays. Lymphocytes from wild-type or *Cd22*^{-/-} donors were injected into wild-type or *St6gal1*^{-/-} recipients, and localization was assessed 1.5 h later. CD22 deficiency had no significant effect on B or T cell homing to PLNs, consistent with a lack of CD22- and ST6GAL1-dependent interactions in PLN HEVs. *Cd22*^{-/-} CD3⁺ T cell homing was largely unaffected even in PPs, consistent with selective B cell expression of CD22. However, *Cd22*^{-/-} B cells homed poorly to wild-type PPs (**Fig. 7c**) and localization of both wild-type and *Cd22*^{-/-} B cells to PPs was severely reduced in *St6gal1*^{-/-} recipients. Although MLNs are more similar to PLNs than to PPs in their B versus T cell recruitment, homing of *Cd22*^{-/-} B cells was also significantly reduced in wild-type MLNs (data not shown). We conclude that high-affinity α 2,6-sialylated glycan ligands for CD22 decorate HEVs in GALT where they function as a B cell selective mucosal vascular addressin.

Discussion

We analyzed the transcriptomes of lymphoid tissue HEV and capillary endothelium from peripheral and GALT, extending previous pioneering studies of mouse LN and human tonsillar HEV gene expression (reviewed¹³). Our results identify transcriptional networks that accompany EC specialization, and reveal expression of cytokine, GPCR, and growth factor receptors that allow CAP and HEV to interpret signals from surrounding immune and stromal cells. They also define tissue-specific gene signatures for HEC, revealing a central role for transcriptional mechanisms in the segmental and tissue specific expression of endothelial determinants for lymphocyte recruitment.

Our results show that lymphoid tissue capillaries express genes and pathways that direct vascular development, and share surface antigens with arterial EC. Although not discussed here in detail, capillaries also display a surprising tissue specialization in gene expression, including unique tissue selective CAP-specific genes, and genes shared with HEV in the same tissue. For example, both capillaries and HEV in GALT expressed NKX2-3, a transcription factor required for MAdCAM1 expression³². Finally, CAP are characterized by

anti-adhesive transcriptional programs, expressing for example the soluble (decoy) LFA1 ligand *Esm1*, as well as glycosylation programs predicted to preclude the synthesis of SLeX-related ligands for leukocyte tethering.

HEV gene signatures 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 prior studies, HEV preferentially expressed *Ccl21* involved in lymphocyte recruitment into lymphoid tissues, and GALT but not PLN HEC highly express *Madcam1* encoding the mucosal vascular addressin.

Blood borne lymphocytes initiate interactions with HEV through L-selectin-mediated “tethering” and rolling. Patterns of expression of genes for glycosyl- and sulfotransferases that control synthesis of L-selectin ligands pointed to a transcriptional basis for the HEV selectivity of L-selectin binding, but also for fine control of the nature of L-selectin ligands. PLN HEV expressed genes predicted to allow synthesis of core 1 as well as core 2 branching biantennary high affinity ligands for L-selectin, correlating with reactivity of the core 1 extension 6-sulfo-LAcNAc-specific PNAd antibody MECA-79. Generation of biantennary 6-sulfo-SLeX ligands may contribute to the high avidity, low velocity L-selectin mediate rolling of lymphocytes in PLN HEV. In contrast, PP HEV showed reduced expression (compared to PLN HEV but not CAP) of genes involved in terminal fucose and sulfate addition to LAcNAc, genes required for high affinity L-selectin ligands. Consistent with previous studies, PP HEV also expressed minimal *Chst4* required to generate core1 L-selectin-binding glycotopes. Together these features correlated with greatly reduced PP HEC reactivity with S2 antibody to 6-sulfo-SLeX, and lack of core 1-specific MECA-79 reactivity. On the other hand, PP HEC stained only 2-3 fold less well than PLN HEC with antibody HECA-452 which binds low as well as high affinity L-selectin ligands including non-sulfated SLeX. Reduced expression of high affinity L-selectin ligands, combined with lack of the enhanced valency provided by biantennary 6-sulfo-SLeX-capped glycans, likely explains the less avid (high velocity) rolling mediated by L-selectin in PP HEV⁴. This loose rolling is critical to the specificity of lymphocyte homing to PP, since it does not slow cells sufficiently to allow chemokine activation of lymphocyte arrest, thus enforcing a requirement for additional “braking” mediated by $\alpha 4\beta 7$ interaction with MAdCAM1^{4, 49}.

Selective expression of *St6gal1* in PP HEV led us to uncover a vascular addressin for B cell targeting to GALT. B cells home efficiently to PP in support of mucosal humoral immunity, while in comparison with T cells they home poorly to PLN⁴⁸. In short term homing assays, *St6gal1*^{-/-} mouse PPs were dramatically defective at recruiting B cells from the blood. ST6GAL1 generates ligands for B cell expressed CD22 (Siglec2)³⁸, and we showed that a CD22-Ig chimeric protein selectively binds PP HEV. Moreover, CD22 deficient B cells showed reduced homing to wild type PP. The findings reveal a role for CD22 and ST6GAL1-dependent CD22 ligands in B cell homing to PP. The known preferred ligand for mouse CD22 is NeuGc α 2-6LAcNAc, and PP HEC expressed transcripts for enzymes required for its synthesis including CMAH, which generates NeuGc from NeuAc. Humans lack CMAH, and human CD22 binds 6-sulfo-NeuAc α 2-6LacNAc as a preferred ligand. While the specific structures of CD22 ligands on PP HEV remain to be determined, they are

probably generated by the combined actions of ST6Gal1, CMAH and potentially HEV sulfotransferases: in addition to NeuGc α 2-6LacNAc, 6-sulfo-NeuGc α 2-6LacNAc is a likely candidate. Interestingly, CD22 contributes to accumulation of mature recirculating B cells to bone marrow where sinusoidal EC express unknown CD22 ligands⁵⁰; cytokine activation of EC *in vitro* induces functional CD22 binding⁵¹; and human HEV stain with an antibody to the CD22 ligand 6-sulfo-NeuAc α 2-6LacNAc⁴³, leading 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 in B cell homing to GALT. CD22 is the first Ig family member shown to function as a lymphocyte homing receptor through Ig domain recognition of EC. Other leukocyte Ig family members, in particular other Siglecs, should now be considered candidate receptors for endothelial recognition and leukocyte trafficking.

The results also uncovered HEV expression of molecules implicated in leukocyte-vascular interactions but not previously associated with high endothelium. Bst1, implicated in neutrophil diapedesis in culture models³³, is expressed differently by PLN versus PP HEVs suggesting a role in tissue selective lymphocyte-HEV interactions. CD63 is required for granule (Weibel Palade body) exocytosis and for P-selectin expression following EC activation⁷. HEV expression suggests a potential role in lymphocyte HEV interactions as well. Chemokine scavenger receptor Ackr2, which is expressed by lymphatic endothelium and binds and internalizes inflammatory but not homeostatic chemokines to facilitate resolution of inflammation, is also expressed by HEVs, as shown by our data, suggesting it may also limit inflammatory chemokine presentation by HEV. Our analyses also identified B4GALT5 and 6 as additional candidate HEV glycosyltransferases for synthesis of L-selectin ligands, and revealed segmental and tissue selective expression of sulfate and UDP-fucose transporters involved.

HEV also expressed genes encoding enzymes for metabolism of diverse lipid mediators including eicosanoids, LPA, and sphingosines implicated in both vascular and immune cell function. In the context of lymphocyte migration, studies of S1P have focused primarily on its role in lymphocyte exit from lymphoid tissues into lymph. However, *S1pr1* expression by lymphocytes contributes to interactions with PLN (but not PP) HEV²⁹, an observation that correlates with higher *Sphk1* and *Asah2* in PLN HEV and suggests a role for local S1P production in lymphocyte entry. Autocrine synthesis of S1P may also have unique effects on HEC: while plasma S1p supports EC integrity and barrier function, intracellular S1P or over expression of *Sphk1* in EC reduces cell proliferation and loosens or disrupts cell-cell junctions⁵², features arguably characteristic of HEV. Elucidation of the importance of autocrine HEV expression of S1P will require targeted genetic manipulation of S1P metabolism. Consistent with prior studies^{24, 28}, HEC (but surprisingly also CAP) abundantly expressed transcripts for autotaxin, which generates LPA locally and contributes to lymphocyte recruitment via HEV.

HEV highly expressed transcripts for Ch25h which synthesized 25-OHC, a sterol involved in lipid metabolism and immune activation⁵³. 25-OHC is the immediate precursor of 7 α , 25OHC, the most potent known attractant for the lymphocyte and dendritic cell (DC) chemoattractant receptor Gpr183. The 7 hydroxylase CYP7B1 required for generation of the

active attractant is expressed by lymphoid stromal FRC^{5, 54}. HEV also expressed the gene for the enzyme that degrades Gpr183 attractants, which could prevent stroma-derived Gpr183 agonists from reaching the vascular lumen. On the other hand, trans-cellular metabolism predicted, with HEV generation of 25OHC and degradation of stromal cell-derived 7 α ,25OHC, could establish of a steep gradient of the agonist to attract Gpr183 - expressing lymphocytes and DC away from HEV and into the surrounding tissue. The role of Gpr183 in lymphocyte recruitment via HEV has not been examined.

Mucins play important roles as acceptors of glycotopes for lymphocyte interaction and repulsion. Our data show that *CD34*, *PODXL*, *Glycam1*, and *MAdCAM1* display pan-EC, capillary-, HEV- or GALT HEV-selective expression, respectively, correlating with reported protein expression. While their function as pro- or anti-adhesive functions depends on the nature of their carbohydrate modifications, their EC subset specific expression suggests that mucins may have specialized roles *in vivo*, perhaps relating to differences in glycosyltransferase substrate preferences. In addition to previously described mucins, we identify Parm1 as a novel HEV-specific mucin that is preferentially expressed in PLN, and show that it is decorated by PNAd glycotopes and thus likely contributes to L-selectin mediated homing as well.

Not all genes expressed in BEC correlate with protein expression. As examples, *Vcam1* and genes for E- and P-selectin are high in HEV, and *Stab1* in all BEC, while the adhesion receptors they encode are displayed minimally or undetectably on lymphoid tissue BEC in the mouse²⁶. HEV may utilize post-transcriptional mechanisms to regulate these inflammation- and lymphocyte migration-associated adhesion receptors.

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

METHODS

Reagents

Anti-CD31 PE-Cy7 (390), anti-CD45 PerCP-Cy5.5 (30-F11), anti-Gp38 PE (eBio8.1.1), and anti-Ter-119 PerCP-Cy5.5 (TER-119) were purchased from eBioscience. Anti-CD3 PE-Cy7 (145-2C11), anti-CD19 APC-Cy7 (6D5), anti-IgD APC (11.26c.2a), anti-CD326 PerCP-Cy5.5 (G8.8), anti-CD11a PerCP-Cy5.5, (H155-78), and anti-GFP (FM264G) were purchased from Biolegend. Anti-mouse Parm1 (EPR10009) was purchased from Abcam. Donkey anti-Rat IgG Dylight488, donkey anti-goat IgG Dylight488, and donkey anti-Rabbit IgG Alexa488 were purchased from Jackson ImmunoResearch Laboratories, Inc. HECA-452, MECA-79, MECA-367, MECA-99 were produced in our lab from hybridomas and labeled using DyLight Antibody Labeling Kit (Thermo Fisher Scientific) with fluorophores indicated. Goat F(ab)2 anti-human IgG PE, Carboxyfluorescein succinimidyl ester (CFSE) and Celltracker Violet were purchased from Invitrogen. Collagenase P and

Dispase II, neutral protease, grade II were purchased from Roche. DNase I from bovine pancreas was purchased from Sigma. FITC labeled Sambucus Nigra (SNA) lectin was purchased from Vector laboratories. Polyclonal goat anti-mouse Nrpl and mouse CD22-Fc fusion proteins were purchased from R&D systems. All reagents were titrated or used according to the manufacturers' recommendations. Antibodies used for immunoprecipitation and immunoblotting are described below.

Mice

6-8 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. In some immunofluorescence staining, Hes1-EmGFP^{SAT55} mice age 6-month-old male and female were used. These mice were bred and maintained in the approved animal facilities of Veterans Affairs Palo Alto Health Care System. For lymphocyte trafficking studies, WT, *Cd22*^{-/-} ⁵⁶ and *St6gal1*^{-/-} ⁵⁷ mice on the C57BL/6J background were bred and housed at Scripps Research Institute. All animals were maintained and bred in specific pathogen-free conditions in the animal facility of Veterans Affairs Palo Alto Health Care Systems or Scripps Research Institute, and all animal work was approved by the IACUC 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 endothelial cells for flow cytometry

Peripheral (inguinal, axillary and brachial) LNs, mesenteric LNs and PPs were carefully isolated from equal numbers of male and female BALB/c mice. Tissues were lightly minced in ice-cold HBSS and collected by 2 min centrifugation at 100g 4 °C. For PP preparation, additional wash steps (12 × 5ml ice-cold HBSS washes using 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 in HBSS media containing 0.2 mg/ml collagenase P, 0.8 mg/ml Dispase II, 0.01 mg/ml DNase for 60 min at 37 °C with gentle rocking. Digestion was stopped by adding FBS (30% final concentration) on ice. Dissociated cell suspensions were then passed through 100 µm filter follow by 40 µm filter. HECs and CAP were enriched from the resulting cell suspensions by depletion of hematology cells with anti-CD45 mouse MicroBeads (Miltenyi) following the manufacture's protocol. Enriched endothelial cells were labeled with antibodies for flow cytometry. The cells were stained in with the indicated fluorochrome-conjugated antibodies or with CD22-Fc protein in HBSS containing 2% FBS using standard protocols. Dead cells were excluded by propidium iodide staining (Sigma). Background fluorescence levels were determined by Fluorescence Minus One (FMO). Flow cytometry data was acquired on either a LSRII or a Fortessa (BD), using Diva software (BD). Further analysis was performed using FlowJo from Treestar.

Microscopy

Peripheral (inguinal, axillary and brachial) LNs and PPs were isolated from male and female BALB/c mice and placed in Tissue-Tek (Sakura) and frozen at -80 °C. Cryosections of acetone-fixed or 4% paraformaldehyde-fixed tissue were stained following standard

protocols with the indicated antibodies. Staining was imaged on an LSM 710 confocal microscope (Carl Zeiss).

Immunoprecipitation

Peripheral (inguinal, axillary, and brachial) and/or mesenteric LNs were isolated from male and female C57BL/6J mice, and membrane enriched protein fraction was produced using the ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem) according to manufacturer's instructions. Rabbit monoclonal antibody for Parm1 (1/100) and Protein G Dynabeads (Life Technologies) were used to immunoprecipitate Parm1 from the membrane enriched protein fraction. Serving as an immunoglobulin control, anti-Hsp90 [C45G5], a monoclonal rabbit IgG (1/100, Cell Signaling Technology) was also used for immunoprecipitation. Immunoprecipitates were denatured in SDS loading dye, separated on 7.5% SDS-PAGE and transferred to PVDF membrane. Immunoblotting was completed using anti-Parm1 (1/1000) and MECA-79 (2 µg/ml) primary antibodies and donkey anti-Rabbit IRDye 800CW (1/5000, LI-COR) and Alexa Fluor 680 conjugated goat anti-Rat IgM (1/10000, Jackson ImmunoResearch) secondary antibodies, respectively. Images were acquired using the LI-COR Odyssey CLx infrared imaging system.

Microarray Analysis

Endothelial cells were enriched by anti-CD45 depletion as above from equal numbers of 6-8 week old BALB/c male and female mice, typically 10-14 mice per experiment. Tissue processing time (from sacrifice the first mouse to start sorting) was generally less than 4 h. Enriched ECs were stained with the indicated antibodies and propidium iodide to exclude dead cells, and sorted on a BD FACS Aria III (100 µm nozzle, BD Biosciences) with flow rate setting between 1-2 (fewer than 2500 events per second). Monoclonal antibodies to gp38 (podoplanin), CD31, and lineage markers were used as described⁵ to define BECs (lineage-negative CD31⁺gp38⁻; blood endothelial cells) and to separate BECs from other cell types (lymphatic endothelial cells, fibroblastic reticular cells, hemolymphoid cells). Lineage markers for exclusion included CD45 to eliminate most hemolymphoid cells, Ter119 to eliminate erythrocytes, LFA1 to eliminate plasma cells, and EpCAM to eliminate epithelial cells. Gated BECs were further sorted using PNAd-specific antibody MECA-79 and/or mucosal vascular addressin (MAdCAM1)-specific antibody MECA-367 to define HEV, and 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 identified in the submitted microarray data, but because of the similarity of their overall gene expression all MLN samples were pooled for the analyses performed 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 (representative plots in **Supplementary Fig. 3**).

RNA was isolated from the sorted BEC subsets using Qiagen's RNeasy Plus Micro kit. 5-20 ng of total RNA from each sample (RNA Integrity Number at least 8, as determined by Agilent bioanalyzer at the Stanford University PAN facility (Stanford, CA)) was used for amplification, labeling, and hybridization which were carried out by Expression Analysis, Inc (Durham, NC). Hybridization was performed on Affymetrix Genechip® Mouse Gene

1.0 ST Array. GeneSpring GX 12.6 and Partek Genomic Suite (6.6) were used for processing and analyzing the data. Genespring preprocessing and default normalization (RMA-16) were applied.

Quality control determination was performed as described by Immgen Consortium⁵. Specifically, for all samples analyzed in this study, positive versus negative AUC (area under the curve) values were at least 0.8 and dynamic range at least 40 (average of all 19 samples is 59 with standard error of 1). Significant cellular contamination was excluded by evaluating transcripts of genes known to be highly expressed by potential contaminating cell populations: for T cells, *Tcf7*, *Fyb*, *Lat*, *Thy1*, and *Cd3g*; for B cells, *Igk*, *Cd79b*, *Igh-6*, *Ms4a*, *Pax5*, *Igj*, and *Igk*; for epithelial cells, *Krt19*, *Krt1*, *Epcam*, *Muc1*, and *Cdh1*; for myeloid cells, *Anxa3*, *Alox5ap*, *Il13ra1*, *Tlr13*, and *Il13ra2*; for platelets, *Gp1ba*, *Itga2b*, *Mpl*, and *Gp9*, and *Epor*; for red blood cells, *Hba-a1*, and *Hba-a2*; for sign of cellular stress, *Hspa8*. Cellular purity levels for all samples described here are similar to those of stromal cell samples in the Immgen Consortium⁵.

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

In addition to capillary EC, MECA-99 stains arterial EC (M.D.L. and E.C.B. unpublished data). The percentage of MECA-99⁺ endothelium within lymphoid tissues that is within arteries was estimated as ~5% as determined morphometrically in multiple sections of PLN and PP with the aid of elastin binding fluorescent dye⁵⁸ to define arterial endothelial cells and to distinguish them from capillary endothelium. We therefore refer to sorted addressin-negative MECA-99⁺ BECs as capillary ECs (CAP) throughout the manuscript.

Additional analysis was performed to evaluate PP CAP samples due to the possible contamination from endothelium from non-PP small intestine. MECA-367⁺ MECA-99⁻ BECs were too rare or weakly positive for MECA-367 reactivity for detection among ECs isolated by digestion of PP-depleted intestine samples (data not shown). Briefly, PPs were clipped carefully from small intestines, minimizing the inclusion of non-PP gut wall (as performed when preparing samples for microarray analyses). Sections of multiple PPs were analyzed morphometrically using an ocular grid to determine the percent of lymphoid tissue vs non-lymphoid lamina propria and muscularis. The area of PPs was defined by immunofluorescence staining for B and T cells (anti-IgD and CD3). Morphometric analyses indicated that ~78% (SEM 1% from at least 6 PPs per mouse; PPs from 2 mice were analyzed) of the isolated tissues comprised PP lymphoid tissue. Moreover, the recovery of CAP from PPs was significantly more efficient than from non-PP intestines with our isolation protocol: ECs were dissociated enzymatically from similar sized fragments of PP-depleted small intestine as outlined above and enumerated by flow cytometry. Twice as many ECs were recovered from PPs as from non-PP gut wall (per unit wet weight; $n = 2$). From these analyses, we estimated ~12% contamination of PP lymphoid tissue CAP with MECA99⁺CD31⁺ BECs 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 signals from contaminating cells cannot be formally excluded. However, in addition to ruling out significant contamination of our sorted cells by other characterized lymphoid tissue cells types (by evaluation of the cell-specific marker genes discussed above), we accessed other data sources to evaluate EC expression of the top 5 most differentially expressed genes in HEV or CAP signature gene sets, and in PLN versus PP HEV signature genes (i.e. genes from heatmaps in **Fig. 2b** and **Fig. 5a**). In most cases, gene expression by cultured BECs, or immunohistochemical confirmation of expression by BECs *in vivo*, was reported in the literature. For other genes, we evaluated endothelial gene expression in public datasets. We analyzed deposited data from cultured human or mouse BECs, and considered expression within the top 25% of genes as indicating significant EC expression. We also took advantage of Immgen consortium datasets to assess 1) expression of the test genes by sorted mixed blood endothelial cells from PLNs and MLNs in independent studies from C57BL/6 mice, 2) B and T lymphocytes and dendritic cell subsets; 3) lymphatic endothelial cells; 4) fibroblastic reticular cells; and 5) “double negative” stromal cells that are enriched in pericytes⁵. Together the Immgen stromal datasets encompass all dissociated stromal (CD45 negative) cells released enzymatically⁵. Most test genes were highly expressed by total BECs in the Immgen database, and many genes were more highly expressed in BECs than in any other Immgen defined stromal subset in PLNs or MLNs, or in lymphocytes, DCs or macrophages. Endothelial expression of all “top 5” signature genes was supported by one or more of these criteria. Together, these considerations suggest that most highly differentially expressed genes in our analyses are expressed by the target EC subsets themselves.

Interestingly, however, four genes expressed by cultured ECs and highly expressed in our samples were only weakly or not expressed in the Immgen lymph node BECs, even though these BECs should comprise a mixture of CAP and HECs. *Tc2n*, *Tshr*, *Pf4*, and *Fjx1*, highly expressed in our sorted HEVs from male and female BALB/c mice, were not or only very weakly expressed (EV<120) in Immgen LN BECs, which were from male C57BL/6 mice. These results suggest significant strain-specific expression of BEC genes, although sex differences are also possible.

Short-term homing Assays

Donor splenocytes were isolated from either WT or *Cd22*^{-/-} mice and labeled with Celltracker Violet (CTV) or CFSE. Labels were alternated in different experiments to rule out potential effects of labeling on cell behavior: under the condition employed, the CFSE and CTV labeled cells behaved indistinguishably *in vivo*. 60 million (30 million cells each from WT or *Cd22*^{-/-} mice) labeled cells were then injected into WT or *St6gal1*^{-/-} recipients via tail vein injection. After 1.5 h, lymphocytes from peripheral (inguinal, axillary and brachial), mesenteric LNs, and Peyer's patches of recipient mice were isolated, stained with antibodies to CD3, CD19 and IgD to define T and B cell subsets, and analyzed by flow cytometry. Within each experiment, the homing of IgD⁺ B cells and CD3⁺ T cells from WT and *Cd22*^{-/-} donors was evaluated. Results are presented as relative localization ratios (RLR)⁴⁸, which are calculated by normalizing the efficiency of homing of each subset to that of WT CD3⁺ T cells in each organ.

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

Results were pooled from 4 independent homing assays. In 2 sets of recipients WT cells were CFSE labeled and *Cd22*^{-/-} cells were CTV labeled, and in 2 others the labels were reversed. No effect of the labels on homing was observed.

Statistical analysis

The statistical significance of differences between sets of data was assessed by two tailed unpaired Student's *t*-test unless stated otherwise. Error bars shown indicate standard errors unless otherwise indicated. Analytic methods for significance of differential gene expression are indicated in the text. Significance of clusters was determined by multiple bootstrap resampling⁵⁹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Isolation and transcriptional diversity of lymph node and Peyer's patch blood endothelial cell subsets. **(a)** Flow cytometry gating strategy for isolating HEVs and CAPs from lineage-negative CD31⁺ gp38⁻ BECs of PLNs and PPs. Numbers in blue indicate the percentages of cells within the gates. PLN HEV marker, peripheral lymph node addressin (PNAd)-specific antibody MECA-79, and PP HEV marker, mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1)-specific antibody MECA-367. CAP marker, antibody MECA-99. **(b)** Representative immunofluorescence staining of PLN and PP cryosections with

antibodies used for sorting BECs in **a**. HEV makers (*green*), MECA-79 and MECA-367. CAP marker (*red*), MECA-99. Representative of more than 5 independent experiments. Scale bar is 100 μm . **(c)** Principal component analysis of HEC and CAP transcriptomes from PLNs, MLNs, and PPs, calculated for normalized expression values of genes that are at least 2 fold different ($P < 0.05$ by one-way ANOVA) between any pair of samples and with raw expression (EV) >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. Each data point represents a biological replicate of the gene expression profile of the indicated sample cell type sorted from pooled tissues from 10 to 14 BALB/c mice, aged 6-8 weeks and comprising equal numbers of males and females. **(d)** Hierarchical clustering by correlation of PLN, MLN, PP HEC and CAP samples with the gene list defined in **c**. The heatmap is colored based on $\log_2$ normalized EV as indicated in the figure. Each terminal branch represents results from a single microarray performed on an independent biological replicate of sorted cells as described in **c**. Significance of clusters determined by multiple bootstrap resampling, * $P < 0.01$ and ** $P < 0.05$.

Fig. 2.

HEV and capillary endothelial cell (CAP) gene signatures. **(a)** Venn diagrams showing counts of genes differently expressed in HEC versus CAP (1.5 fold different; and EV >140 in all samples of HEC or of CAP within an organ). Genes up in HEC (left) or in CAP (right) in all three sites are in white, and are defined as HEV or CAP signature genes. **(b)** Top 100 differently and highly expressed HEC and CAP signature genes from **a** (4 fold change CAP vs HEV in at least one tissue, and EV >900 in one or more of the samples). Genes are ordered and colored in the heatmap based on log₂ normalized EV as indicated in the figure.

Blue gene symbols indicate that the mean EV of replicates for at least one of the cell types is less than 140. Numbers on the right indicate the highest mean EV among the cell types. **(c)** *Left*: expression ($\text{EV} \times 10^{-3}$) of *Cd63* in individual HEC and CAP samples from PLN, MLN, and PP, shown with mean and standard error (SEM). *Right*: FACS plots of isolated BEC stained with CD63-specific antibody and gated on CAP (MECA-99⁺; MECA-79⁻ for PLN or MECA-367⁻ for PP) or HEV (MECA-99⁻; MECA-79⁺ for PLN or MECA-367⁺ for PP) as indicated. Results are representative of 2 independent experiments of pooled tissues from 4 BALB/c mice each. Light and dark grey represent the background staining for PLN and PP, respectively. Red and Blue lines represent antibody staining, and numbers indicate specific mean fluorescence intensity (mean fluorescence intensity after background value subtraction) for PLN and PP EC samples. **(d)** *Left*: expression ($\text{EV} \times 10^{-3}$) of *Ly6c1* in individual HEV and CAP samples from PLN, MLN, and PP, shown with mean and SEM. *Right*: FACS plots of HEC and CAP stained with anti-Ly6C as described in **c**. Results are representative of 2 independent experiments of pooled tissues from 4 BALB/c mice each.

Fig. 3.

Gene ontology (GO) and pathway analyses of HEV and CAP signature genesets. **(a)** Selected GO terms and pathways that are significantly enriched ($P < 0.05$, using Benjamini Yekutieli correction in Genespring) in the signature genesets described in **Fig. 2a**. Ontology terms are ordered and colored in the heatmap based on negative $\log_{10}$ of the corrected P values. **(b)** Scatter plots showing mean EVs in HEC versus CAP for genes belonging to the indicated GO terms. Labeled genes with large dots are HEC or CAP signature genes (fold change >1.5 between HEC and CAP, $P < 0.05$). Mean EVs are the average of all HEC

samples (3-4 each from PLN, MLN and PP), or all CAP samples (2 from MLN and 3 from PLN and PP). Gray dashed lines are the 1.5 fold difference cut off. Numbers on axes are $\log_2$ EVs. **(c)** Representative immunofluorescence histology of PLN from 8 week old BALB/c mice illustrating CAP-selective expression of Nrp1. PP sections show similar pattern (data not shown). Data are representative of 3 independent experiments. Scale bar is 100 μm . **(d)** Representative immunofluorescence images of PLN from Hes1-GFP transgenic (knock-in) reporter mice with anti-GFP (*green*) and MECA-99 (*red*). PP sections show similar pattern (data not shown). Data are representative of 2 independent experiments. Scale bar is 100 μm .

a

b

c

Fig. 4.

Expression of selected cytokines/chemokines, their receptors, integrins, GPCRs, transcription factors, mucin and Ig domain containing adhesion receptors in HEV and CAP of lymphoid tissues. (a) Heatmap of selected gene families as indicated in the figure (EV > 140 in any of the samples). Genes are colored in the heatmap based on log₂ normalized expression values as indicated. Blue gene symbols indicate the mean EV for at least one of the cell types is less than 140. Numbers on the right indicate the highest mean EV among the cell types. (b) Expression (EV × 10⁻³) of genes for selected Ig family, mucin domain,

selectin, and other adhesion receptors, and of selected enzymes implicated in phospholipid and oxysterol metabolism. The EV for each biological replicate is plotted with mean and SEM. (c) Representative Immunofluorescence images of PLN cryosections stained with anti-Parm1 (*red*) and MECA-79 (*green*). Data are representative of 3 independent experiments. Scale bar is 100 μ m. Enlarged insert illustrates HEV surface detection of Parm1 (yellow arrow).

Bst1 as described in **Fig. 2c**. Results are representative of 2 independent experiments using pooled tissues from 4 BALB/c mice each. (c) Selected GO terms and pathways that are significantly enriched ($P < 0.05$, using Benjamini Yekutieli correction in Genespring) in the signature genesets described in **a**. Ontology terms are ordered and colored in the heatmap based on negative $\log_{10}$ of the corrected P values.

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Fig. 6. Segmental and tissue selective expression of genes involved in the synthesis of L selectin ligands. **(a)** Diagram showing the structure of core 1 and core 2 6-sulfo-SLeX, and recognition determinants for L-selectin, MECA-79, HECA-452, and S2. Genes for enzymes involved in sequential synthetic steps are indicated in black. *St3gal4* (red) is expressed but not implicated in lymphocyte homing. Numbers with gray arrows indicate the fold difference in EV between PLN HEV versus PP HEVs for selected genes. **(b)** Scatter plots comparing average EV for glycosyltransferases and related genes in the indicated BEC

populations. Large dots indicate the glycosyltransferases and related genes that are significantly different (fold difference >1.5 and $P < 0.05$) between the 2 plotted populations in each graph. Gray dashed lines are the 1.5 fold difference cut off. Numbers on axes are $\log_2$ EVs. **(c)** FACS plots of MECA-79, HECA-452 or S2 staining of isolated HEC as described in **Fig. 2c**. Results are representative of 2 independent experiments with pooled tissues from 4 BALB/c mice each. Similar results were obtained in experiments in which HEC were stained with MECA-79, HECA-452 or S2 separately rather than in combination (1 additional independent experiment for each of the three antibodies listed here). **(d)** Representative immunofluorescence staining of PLN and PP cryosections with MECA-367 (*green*) and S2 (detecting 6-Sulfo-SLeX, *red*). Side by side staining illustrates strong S2 staining of PLN compared to PP HEV. Data are representative of 3 independent experiments. Scale bar is 100 μm .

Fig. 7.

Selective PP HEC expression of *St6gal1* confers CD22 recognition and CD22-mediated B cell homing to GALT. (a) Diagram showing the structure of 6'-sialyl-LacNAc, the minimal recognition determinants for SNA lectin, and possible structures of high affinity PP HEV CD22 ligands (NeuGcα2-6Galβ1-4GlcNAc or sulfated NeuGcα2-6Galβ1-4[6S]GlcNAc). Genes for enzymes involved are indicated in black. Sulfate is in gray since the existence of sulfated CD22 ligands in rodents is speculative. Numbers with gray arrows indicate the fold difference in EV between PLN HEV versus PP HEVs for selected genes. (b) Left:

Expression ($\text{EV} \times 10^{-3}$) of *St6gal1* in individual HEV and CAP samples from PLN, MLN, and PP, shown with mean and SEM. *Right*: FACS plots of SNA and CD22-Fc staining of HEC and CAP as described in **Fig. 2c**. Results are representative of 2 independent experiments with pooled tissues from 4 BALB/c mice each. Similar results were obtained for CD22-Fc staining of PP HEC from C57BL/6J mice (2 independent experiments with HEC pooled from 4 mice each). **(c)** Histogram showing short term (1.5 h) homing of WT and *Cd22* deficient donor B and T cells in WT and *St6gal1* deficient recipients. CFSE or CTV labeled donor cells isolated from WT and *CD22*^{-/-} spleen were injected together into either WT or *St6gal1*^{-/-} mice. PLN, MLN, PP, and spleen were then isolated from recipient mice and stained with antibodies for CD3, CD19, and IgD to quantify B and T cell localization. Data are presented as relative localization ratios (RLR), which represent the efficiency of homing of each subset to the indicated organ expressed as a ratio to the homing efficiency of WT CD3⁺ T cells to the same organ (see Methods). The RLR of WT CD3 cells in each site is thus unity. Data are pooled from 4 biological replicates (4 recipient mice for WT and 4 for *St6gal1*^{-/-} recipient groups) carried out in 2 independent experiments using fluorescence cell labeling. Similar results were obtained in an additional experiment that employed CD45 allotypic markers to monitor B and T cell recruitment (not shown). Bars indicate mean with SEM. * indicates significant difference from WT B cell homing into control PPs ($P < 0.01$, 2 sided paired T test).