Trends in Immunology

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Review

High endothelial venules as potential gateways for therapeutics

Bilal Hussain,[1,6] Vivek Kasinath,[1,6] Gabriel P. Ashton-Rickardt,[2] Thomas Clancy,[3] Kenji Uchimura,[4] George Tsokos,[5] and Reza Abdi[1,*]

High endothelial venules (HEVs) are specialized blood vessels that support the migration of lymphocytes from the bloodstream into lymph nodes (LNs). They are also formed ectopically in mammalian organs affected by chronic inflammation and cancer. The recent arrival of immunotherapy at the forefront of many cancer treatment regimens could boost a crucial role for HEVs as gateways for the treatment of cancer. In this review, we describe the microanatomical and biochemical characteristics of HEVs, mechanisms of formation of newly made HEVs, immunotherapies potentially dependent on HEV-mediated T cell homing to tumors, and finally, how HEV-targeted therapies might be used as a complementary approach to potentially shape the therapeutic landscape for the treatment of cancer and immune-mediated diseases.

Drug delivery in the treatment of cancer and immune-mediated disorders

Current treatments for many types of cancer and immune-mediated disorders, such as transplant rejection, are plagued by middling efficacy and off-target toxicity. Therefore, the development of delivery platforms that concentrate the activity of therapeutics at their intended sites is of utmost importance [1]. One such method that has shown increased potential for success in preclinical studies is the use of blood vessels called HEVs as selective gateways for the delivery of therapeutics to LNs, which are key sites of the activation and regulation of acquired immunity. We posit that the discussed selective drug delivery method might provide an essential step forward in the quest for precision medicine in the treatment of malignancies and certain immune-mediated diseases.

Distinctive morphological and biochemical features of HEVs

Under healthy conditions, mammalian HEVs are specialized blood vessels found in **secondary lymphoid organs (SLOs)** (see Glossary) such as the LNs and tonsil, but not the spleen. LNs are sites in which immune responses are initiated and maintained to generate protective immunity against exogenous pathogens and tolerance to self-antigens and commensal organisms [2]. HEVs are also found in tertiary lymphoid organs (TLOs), which are LN-like immune cell clusters that develop in non-lymphoid organs in response to chronic inflammation, stimulated by persistent infections, chronic graft rejection, autoimmunity, and cancer [3]. HEVs are readily distinguishable from other blood vessels by a multitude of histological and biochemical markers (Figure 1) [4]. High endothelial cells (HECs) have a characteristic plump morphology, and the endothelial layer of HEVs is surrounded by a thickened basement membrane, which is more pronounced than in other types of blood vessels. HEVs are supported by a perivascular sheath formed by fibroblastic reticular cells (FRCs) that is connected to the FRC-coated conduit system in the LN. This conduit system allows communication between afferent lymph vessels and the HEVs, whereby incoming lymph-borne soluble factors, such as chemokines and cytokines, are directly delivered into the basal lamina of the HEV [4].

Significance

Certain drugs against cancers or immune-mediated diseases are marked by limited efficacy and off-target toxicity; however, targeting high endothelial venules for selective nanocarrier-based, active drug delivery harbors significant potential to solve this clinical problem.

Highlights

High endothelial venules (HEVs) are specialized mammalian segments of vasculature that are specific to secondary lymphoid organs and hence represent potential targets for immunotherapeutics.

Antibodies that bind selectively to HEVs provide efficient targeting tools for immunotherapeutics through conjugation or encapsulation inside HEV antibody-coated nanocarriers.

HEVs can also develop in organ tissues in association with autoimmune and immune-mediated disorders, including transplant rejection; they are also often found in conjunction with tertiary lymphoid organs in humans and mice.

Located proximal to primary tumors, tumor-draining lymph nodes, and metastatic lesions in humans and mice, tumor-associated HEVs provide a rationale for the development of potential HEV-targeted, drug-specific delivery to cancer patients.

[1]Transplantation Research Center and Division of Renal Medicine, Brigham and Women's Hospital, Boston, MA, USA
[2]Brandeis University, Waltham, MA, USA
[3]Division of Surgical Oncology, Brigham and Women's Hospital, Boston, MA, USA
[4]University Lille, CNRS, UMR8576 – UGSF – Unite de Glycogiologie Structurale et Functionelle, 59000 Lille, France

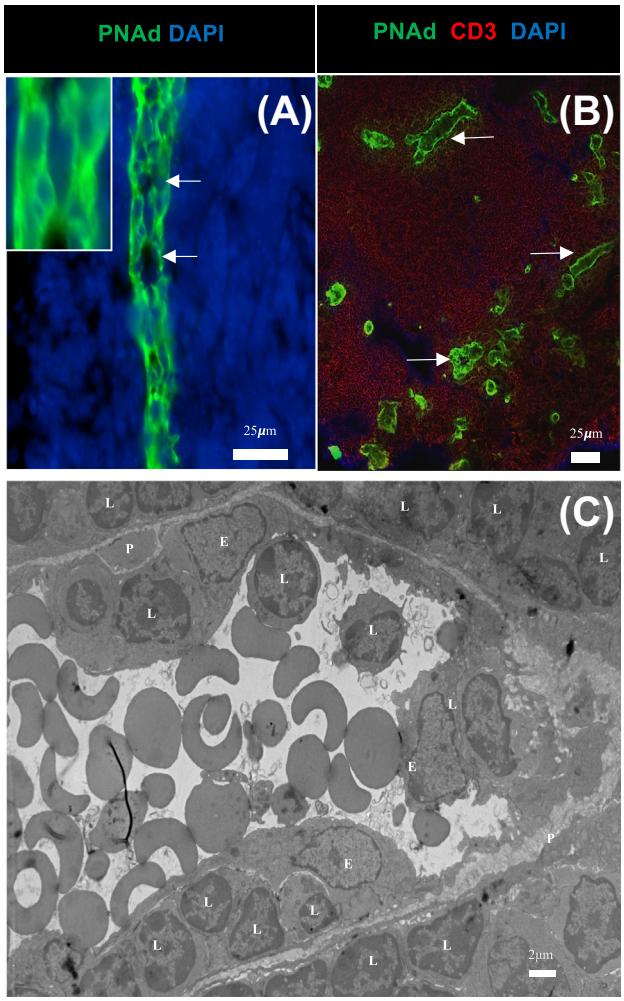

Figure 1. Morphological features of high endothelial venules (HEVs). (A) Fluorescence micrograph of peripheral node addressin (PNAd)$^+$ HEV (green) in the mouse lymph node (LN) demonstrates the thickness and the presence of the characteristic pockets (arrows) of the HEV (nuclear stain DAPI; blue). (B) Fluorescence micrograph displays HEVs (green, arrows) in the paracortex (T cell zone) of the mouse LN, surrounded by CD3$^+$ T cells (red). (C) Electron micrograph of HEV in mouse LN shows orientation of lymphocytes (L), high endothelial cells (HECs) (E), and pericytes (P).

[5]Division of Rheumatology, Beth Israel Deaconess Medical Center, Boston, MA, USA
[6]These authors contributed equally to this work.

*Correspondence:
rabdi@rics.bwh.harvard.edu (R. Abdi).

HECs contain a prominent Golgi apparatus and a greater number of secretory vesicles than other endothelial cells [4]. HECs also express higher concentrations of nonspecific esterase, lactate dehydrogenase, and NADH reductase [4]. Last, biochemical studies have identified a unique biosynthetic pathway for inorganic sulfate in HECs that is crucial for the generation of **peripheral node addressin (PNAd)** and involves a series of important adhesion molecules that mediate L-selectin-dependent binding of lymphocytes to HECs [4]. GlyCAM-1, CD34, sbp200, podocalyxin, endomucin, and nepmucin are all members of this group [5]. The tetrasaccharide sialyl-Lewis X (sLe^X) with *N*-acetylglucosamine (GlcNAc) 6-sulfation (6-sulfo sLe^X) in PNAd is a major ligand for L-selectin [6].

Recent advances in HEV targeting

Development of monoclonal antibodies targeting HEVs

Several monoclonal antibodies have been developed that specifically bind to HEVs. These include MECA-79 [7] and MECA-367 [8], which recognize PNAd and mucosal addressins, respectively. The HECA 452 antibody recognizes part of the functional sLe^X polysaccharide

epitope in PNAd [9]. Recently, another novel monoclonal antibody called MHA-112 was generated against PNAd on HEVs [10]. Other anti-6-sulfo glycan antibodies also recognize 6-sulfo sLe^X and stain HEVs (Table 1). MECA-79, the most widely used antibody in this group, reacts with the PNAd sialomucins through the recognition of a component of 6-sulfo sLe^X present in core 1-extended *O*-glycans with a requirement for GlcNAc 6-sulfation [11]. Two HEV-expressed sulfotransferases, GlcNAc6ST-1 (CHST2) and GlcNAc6ST-2 (CHST4), are essential for the expression of this MECA-79 epitope and L-selectin ligand activity in HEVs [12,13]. Moreover, PNAd, as defined by MECA-79 staining, is also expressed on HEVs at several sites of human chronic inflammation, such as the synovia during rheumatoid arthritis [14–16], the intestine during ulcerative colitis [17–19], and the stomach during *Helicobacter pylori*-associated gastritis [20–22]; this type of staining signals the formation of TLOs. The same two sulfotransferases also underlie the formation of functional PNAd [23,24] at CXCL13-expressing pancreatic islets of RIP-BLC mice [23], the intrapulmonary airways of asthmatic sheep [25], and the synovia from human rheumatoid arthritis patients [23,24].

Transcriptional profiling of HEVs

The development of **single-cell RNA-seq (scRNA-seq)** has permitted scientists to assess and analyze the heterogeneity of immune cells. In one study, full-length scRNA-seq, RNA fluorescence *in situ* hybridization (FISH), and flow cytometry were used to describe the heterogeneity of HEVs in peripheral LNs (PLNs) from adult female C57BL/6J mice [26]. Using scRNA-seq of HECs from the PLNs, the authors reported that the HECs resided in an activated state during homeostasis, as evidenced by upregulation of activity-dependent genes such as *Fos*, *Fosb*, *Jun*, *Jund*, *Egr1*, *Nfkbia*, *Nfkbiz*, and *Zfp36* as well as markers related to splicing, translation, and endoplasmic reticulum (ER) and Golgi processing [26]. They also identified an array of downregulated genes responsible for the differentiated HEV phenotype, such as *Fos*, *Jund*, *Egr1*, *Nfkbiz*, *Gem*, *Dusp6*, *Ppp1r15a*, *Cxcl1*, and *Id2*. In addition, the study showed that LTβR signaling could regulate the HEV **transcriptome** in homeostatic PLNs [26]. Furthermore, several genes deemed responsible for lymphocyte trafficking, including *Glycam1*, *Fut7*, *Gcnt1*, *Chst4*, *B3gnt3*, and *Ccl21a*, were variably regulated after LTβR signaling inhibition in HECs following treatment of C57BL/6J mice *in vivo* with the blocking receptor–antibody fusion protein LTβR-Ig [26]. In the HECs from the LTβR-Ig-treated mice, the expression of the mature HEV genes *Glycam1*, *Fut7*, and *Gcnt1* was markedly reduced, whereas *Ccl21a*, *Chst4*, *Chst2*, *Cd34*, *Emcn*, *Cd300lg*, and *B3gnt3* expression was not affected, compared with those from untreated mice, suggesting that LTβR signaling influenced the capacity of HECs to regulate lymphocyte trafficking [26]. In another study, the transcriptomes of blood vascular endothelial cells (BECs) from PLNs were profiled using scRNA-seq, characterizing various cell phenotypes in the vasculature of mice of mixed genetic backgrounds [27]; specifically, multiple cell subsets were described, such as a medullary

Table 1. Antibodies directed against HEVs

Antibody (species)	Antigen	Refs
MECA-79 (rat antimouse/human)	GlcNAc 6-sulfated oligosaccharide in core 1-extended *O*-glycans on PNAd	[7,75]
MECA-367 (rat antimouse)	Mucosal addressin; not PNAd	[8]
HECA 452 (rat antihuman)	sLe^X oligosaccharide on PNAd	[9]
MHA-112 (mouse antimouse/human)	GlcNAc 6-sulfated sLe^X on PNAd	[10]
G152 (mouse antihuman)	GlcNAc 6-sulfated sLe^X on PNAd	[76]
CL40 (mouse antirodent/human)	GlcNAc 6-sulfated sLe^X and LacNAc in *O*- and *N*-glycans on PNAd	[77]
S1/S2 (mouse antimouse/human)	GlcNAc 6-sulfated sLe^X and LacNAc in *O*- and *N*-glycans on PNAd	[78]

Glossary

Chimeric antigen receptor (CAR) T cell therapy: T lymphocytes that express a genetically engineered CAR. CAR T cells can bind to a specific protein, such as those expressed by malignant cells, and trigger T cell activation. CAR-T therapy has been effective in allogeneic and autologous treatment against certain types of cancer.

Cuboidal high endothelial venule: typical HEV lined by cuboidal HECs that are responsible for facilitating lymphocyte extravasation.

Flat high endothelial venule: HEV subtype found surrounding human melanoma tumors. The presence of this HEV may represent the late quiescent stage of the antimelanoma immune response.

Immune checkpoint inhibitors: examples are PD-1 and CTLA-4, which are extracellularly expressed proteins on T lymphocytes. Activation of immune checkpoints such as PD-1 downregulates T cell activity. Immune checkpoint inhibitors oppose the activity of immune checkpoint proteins, thus preventing T cell downregulation, and have been used to promote antitumor immunity for certain cancers.

INS-GAS transgenic mice: contain the *Ins1-GAS* transgene and overexpress gastrin in the pancreas; well-suited models for gastric cancer.

Peripheral node addressin (PNAd): series of glycoproteins found on the surface of HECs that are ligands for L-selectin expressed by lymphocytes; enable their interaction with HECs. GlyCAM-1, CD34, sbp200, podocalyxin, endomucin, and nepmucin are all members of this group.

Secondary lymphoid organs (SLOs): sites in which immune responses are initiated and maintained to generate protective immunity against exogenous pathogens and tolerance to self-antigens and commensal organisms. These specialized structures include LNs, Peyer's patches, tonsils, and spleen. The blood vasculature in lymphoid organs differs from that found in other organs due to the requirement for efficient recruitment of lymphocytes under non-inflammatory, homeostatic conditions. HEVs perform this function in all SLOs except the spleen.

Single-cell RNA-seq (scRNA-seq): method to isolate single cells, capture transcripts, and generate sequencing

venous population and five capillary subsets, including a capillary resident precursor (CRP) population that harbored transcriptional signatures of both stem cells (*Cxcr4*, *Nes*, *Kit*, *Lnx*, and *Sox4*) and migratory cells (*Cxcr4* and *Ackr3*). Computational predictions of cell alignments suggested that developmental programs were maintained along distinct differentiation trajectories, from CRPs to the formation of mature venous and arterial populations. These alignments recapitulated the microanatomical arrangement of endothelial cells, given that the transcriptomic signatures placed the 'transitional phenotype' capillary epithelial cells between typical capillary epithelial cells and HECs; CRPs in capillary segments; and PNAd$^-$ venous epithelial cells next to HECs [27]. Thus, this study presented a molecular roadmap of the PLN vasculature via scRNA-seq of BECs [27]. Moreover, another transcriptomic analysis of BECs from naive BALB/c mice yielded molecular signatures that distinguished HECs from capillary endothelia and suggested defined tissue-specific HEV specialization, as evidenced by the enrichment of genes related to Golgi and ER function, glycosylation, and lipid and sterol metabolism [28]. HEVs displayed enhanced expression of genes linked to immune defense (*C1s*, *Cfb*, *Cd55*, *Pglyrp1*, and *Hamp*) and lymphocyte homing (*Glycam* and *Cd300lg*), while capillaries expressed gene programs for vascular development (*Esm1*, *Bgn*, *Cxcl12*, and *Cxcr4*) [28].

Together, these studies demonstrated that HECs harbor unique transcriptomic profiles that distinguish them from other BECs in LNs and that LTβR signaling can control the expression of some of these distinctive genes. Future studies are needed to understand how LTβR signaling impacts the formation and expansion of HEVs in LNs, especially in response to immune stimuli.

Inflammation-associated HEVs

Blood vessels that are phenotypically similar to lymphoid tissue HEVs appear in many organs involved in inflammatory diseases in humans, including allergic diseases (asthma [29], allergic rhinitis [30]), organ transplant rejection (acute kidney allograft rejection [31,32], acute heart allograft rejection [33,34]), and other chronic inflammatory diseases, such as rheumatoid arthritis, ulcerative colitis, and gastritis [35]. In rheumatoid arthritis patients, PNAd$^+$ HEV-like blood vessels have been reported to develop in the inflamed synovium and to be associated with GlcNAc6ST-2 (*CHST4*) expression; however, these HEVs can revert to PNAd$^-$ blood vessels following intramuscular administration of anti-TNFα-blocking antibody [14–16]. HEVs have also been shown to form in the intestinal tissues of patients with ulcerative colitis and to be predominantly associated with CD4$^+$ T cell infiltrates; these vessels can also revert during disease remission [17–19]. The same pattern has been observed in the stomach tissues of patients presenting with chronic *H. pylori*-mediated gastritis, given that such HEVs can also revert following eradication of the bacterial infection [20–22]. A similar process of HEV formation has been reported in certain autoimmune pathologies [35], including autoimmune thyroiditis [36], inflammatory skin diseases (e.g., psoriasis, lichen planus, cutaneous lupus erythematosus) [37,38], autoimmune pancreatitis [39], glomerulonephritis [40], and multiple sclerosis [41]. Of note, these inflammation-associated HEVs are often found in conjunction with TLOs [35], although the prognostic significance of these structures in such conditions remains elusive. Understanding the link between the presence or density of inflammation-associated HEVs and the prognosis of these chronic inflammatory conditions could aid pathologists in providing more accurate diagnoses of disease severity, potentially resulting in improved efficacy and selection of treatments.

Tumor-associated HEVs and their putative prognostic value

HEVs also form with malignant solid tumors. By assessing the presence of PNAd$^+$ blood vessels, a study indicated that HEVs were found in the majority of human solid tumors of 319 assessed, including lung, breast, ovarian, and colon carcinomas [42]. These HEVs were associated with the intratumor infiltration of CD3$^+$ and CD8$^+$ T cells, as well as B cells [42]. Analysis of human

libraries in which transcripts are mapped to individual cells.
Transcriptome: collection of mRNA molecules expressed by an organism; also used to define the array of mRNA transcripts produced in a particular cell or tissue type.

breast tumors showed a correlation between tumors with high HEV density and increased T cell infiltration as well as upregulated expression of genes encoding proteins such as CCL19, CCL21, CXCL13, CCR7, and CD62L (associated with T and B cell migration and homing) compared with tumors with low HEV density [42]. The study also demonstrated that high densities of tumor HEVs strongly correlated with low relapse risk as well as improved disease-free survival (~55% vs. ~50%) and overall survival outcomes (~75% vs. ~50%) in breast cancer patients compared with those with low-HEV-density tumors [42]. Of note, retrospective studies have correlated the presence of HEVs in human solid tumors with prolonged patient survival following resection of the primary tumor in several solid cancers such as breast, colorectal, stomach, and non-small cell lung carcinoma (NSCLC) [43]. In some cases and in certain cancers such as melanoma, the density of HEVs alone has been used to help predict patient outcomes [42].

In terms of therapeutic strategies, and because sustained angiogenesis and immunosuppression constitute major hallmarks of cancer, combination treatments targeting these processes have been tested [44,45]. Systemic treatment with the anti-vascular endothelial growth factor receptor 2 (VEGFR2) antibody DC101 and antimouse programmed death-ligand 1 (PD-L1) antibodies induced the formation of HEVs in polyoma middle T oncoprotein (PyMT)-expressing breast cancer lesions in female FBV/n mice, as well as Rip1-Tag2 pancreatic neuroendocrine tumors in transgenic RT2-PNET mice from a C57BL/6 background [46]. Moreover, activation of LTβR signaling in tumor vessels using an agonistic antibody in RT2-PNET pancreatic and PyMT-expressing mammary mouse tumor models enhanced HEV formation and subsequent apoptosis and necrosis of the tumor cells, as well as increased $CD8^+$ T cell infiltration [46]. Treatment with this LTβR agonist also led to the formation of HEVs in a mouse model of recalcitrant glioblastoma multiforme (NFpp10-GBM) and subsequently augmented cytotoxic $CD8^+$ T cell activity at the tumor site, sensitizing the tumor to antiangiogenic/anti-PD-L1 antibody combination therapy in C57BL/6 mice [46]. These findings suggested that the formation of HEVs might potentially augment anticancer immunity, yielding a possible avenue for HEV-mediated approaches to cancer diagnosis and treatment [42].

One study assessed the clinical relevance of TLOs in cancer therapeutics; these structures comprise HEVs and their associated immune cell infiltrates. This work compared efficacy between preoperative neoadjuvant chemoradiotherapy and surgical resection prior to other treatments in human patients with pancreatic ductal adenocarcinoma (PDAC) [47]. Specifically, $PNAd^+$ HEVs and $CD8^+$ T cells in TLOs were greater in number, while programmed cell death 1 $(PD\text{-}1)^+$ lymphocytes were lower in the 'neoadjuvant chemotherapy' group than in the 'surgery first' group. Moreover, the amount of HEV-comprising TLOs per tumor was reported to be an independent positive prognostic factor of malignancy in patients who received neoadjuvant chemotherapy, given that a higher ratio of TLOs per tumor was associated with higher overall survival (~20% vs. 0%). Neoadjuvant chemotherapy was thus associated with a higher density of HEV^+ TLOs in tumors, possibly resulting in better prognosis for PDAC patients, although this remains to be further investigated [47]. Another group analyzed tissue samples from human oral squamous cell carcinoma (OSCC) patients and found that the presence of HEVs correlated with greater $CD3^+$ T cell and $CD20^+$ B cell infiltration and higher CXCL12 and CCL21 but lower CCL20 concentrations compared with tumors without HEVs [48]. The presence of HEVs was also suggested to be an independent positive predictor of higher survival rates, as evidenced by multivariate analysis using Cox's proportional hazards model; these results further implicated HEVs in potentially promoting anticancer immunity, although this still warrants further and robust investigation.

Of note, additional work discussed the role of **cuboidal HEVs** and **flat HEVs** as possible indicators of the respective active and late quiescent phases of tumor regression in human cutaneous

malignant melanoma [49]. Specifically, the density of cuboidal HEVs was positively correlated with lymphocytic infiltration, whereas the density of flat HEVs determined histologically was positively correlated with tumor remission [49]. The samples with greater numbers of cuboidal HEVs than of flat HEVs also harbored greater amounts of CCL19, CCL21, and CCR7, suggesting higher immunological activity, although this hypothesis remains to be further tested [49].

The presence of HEVs has been observed in several different vascularized solid tumors, along with their respective draining LNs and metastatic lesions, in humans [50]. However, the main impact of HEVs in these sites as well as their possible modulation of the immune response remain to be characterized. In addition, the formation of TLOs does not correlate with improved patient outcomes for all cancers. For example, in *N*-nitrosodiethylamine-induced hepatocellular carcinoma in transgenic IKKβ(EE)^{hep} mice that produce TLOs in their livers through constitutive expression of IKK in hepatocytes, TLOs have been reported to promote carcinogenesis through the creation of specific microniches where malignant hepatocytes proliferate and then metastasize [51]. Moreover, in *H. pylori*-infected humans and **INS-GAS transgenic mice** with an FVB background, TLO development has been noted to precede carcinogenesis, but whether TLOs can promote the development of gastric cancer is unclear [52,53]. Recently, surgically resected samples harboring high HEV densities from gastric carcinoma patients were associated with clinical outcomes of longer overall survival, suggesting that the presence of HEVs might be an important prognostic factor for gastric carcinoma, pending further investigation [54].

One group recently highlighted the heterogeneity of tumor-associated HEVs (TA-HEVs) and their adhesion molecules using a methylcholanthrene (MCA)-induced mouse model of cancer [55]. In the same report, metastatic melanoma patients were treated with anti-PD-1 antibody (nivolumab or pembrolizumab) either alone or in combination with an anti-CTLA-4 antibody (ipilimumab), and the presence of TA-HEVs (determined via MECA-79 immunohistochemical staining of skin biopsies) in treated patients predicted better overall and progression-free survival compared with untreated patients [55]. Anti-CTLA-4 monotherapy (but not anti-PD-1 monotherapy) enhanced the number of TA-HECs as well as of terminally exhausted CD8$^+$ T cells in the tumors, pointing to an increased antitumor response after anti-CTLA-4 treatment. These data suggested that the mechanism of anti-CTLA-4 therapy was more dependent on the presence of TA-HEVs and the recruitment of T cells from the periphery than anti-PD-1 blockade, which relies more heavily on the activity of pre-existing T cells in the tumors [55]. The association between the anti-CTLA-4 monotherapy agent ipilimumab and the presence of TA-HEVs is presumably a key consideration [56]. Of note, PNAd$^+$ HEV-related vasculatures have also been found in the TLOs of melanoma, sarcoma, and metastatic renal cell carcinoma tumors of human patients responding to immunotherapy [56–58].

Collectively, these and other studies have sought to determine a correlation between the formation of HEVs and TLOs in tumors and cancer prognosis [47,48], but the nature of this association remains unclear and is likely to vary based on the types of HEVs and the patterns of expression of adhesion molecules on them, as well as other variables including those governing proinflammatory versus anti-inflammatory immune responses in the tumor microenvironment (TME) ('cold' vs. 'hot tumors'). One potential confounding factor in these observational studies is that patients with more benign tumors may survive longer, providing more time for these HEVs to be formed. Future studies to deplete HEVs in the TME to specifically address their effect on cancer prognosis are therefore needed.

HEVs as putative targets for systemically administered therapeutics in cancer and transplantation

The safety of current chemotherapy and immunotherapy drug regimens is commonly hampered by toxicity to off-target organs. Therefore, an unmet need exists for a selective method of

systemic delivery of drugs to cancer lesions. One platform that might provide this capacity is active drug delivery by antibody-conjugated particles, such as microparticles (MPs) and nanoparticles (NPs), and antibody–drug conjugates (ADCs). The major advantages of active drug delivery include stabilization of the drug in serum, extension of its circulation time, and its controlled release from the particles over time [59]. The specificity conferred by antibody–drug conjugation would ideally result in delivering a drug to its intended target and avoiding drug release at off-target sites, thereby limiting toxicity and side effects [60]. However, on the one hand, the drawback of MPs is their large size (diameters in the micrometer range), as many will undergo sequestration in the lung prior to circulating towards the intended target organ [60]. On the other hand, NPs less than 5.5 nm in size undergo swift filtration by the kidney, thereby potentially limiting their circulation time [61]. In this section, we discuss how nanotherapeutics has been harnessed for targeted treatment of various cancers and for the modification of responses in immune-mediated conditions [62]. Since TA-HEVs may play a fundamental role in lymphocyte entry into tumors, they can be explored as putative targets for lymphocyte-mediated cancer immunotherapy, including **immune checkpoint inhibitors**, **chimeric antigen receptor (CAR) T cell therapy**, and various immunomodulators, monoclonal antibodies, and experimental treatment vaccines [35,63].

Recent research has identified the Cys-Gly-Lys-Arg-Lys (CGKRK) peptide as a promising candidate tool to specifically target the tumor vasculature in patients with GBM [64]. This tumor vessel-homing peptide was fused to LIGHT (TNFSF14), a TNF superfamily member, and injected intravenously into orthotopic GBM models in C57BL/6 mice [64]. After treatment, the tumor vasculature appeared to reacquire normal features, such as increased endothelial barrier integrity, pericyte contractility, and tumor perfusion. Moreover, CGKRK–LIGHT induced the formation of HEVs, associated with increased T cell infiltration in solid tumors. Combining CGKRK–LIGHT with antiangiogenic treatment (an antimouse VEGF B20 biosimilar) and immune checkpoint blockade (antimouse PD antibody) boosted HEV induction (based on histological PNAd expression) and cytotoxic T cell infiltration, leading to a reduction in tumor burden [64].

A study from our group illustrated the ectopic formation of HEVs in PDAC and the use of MECA-79-conjugated NPs (MECA-79-NPs) to deliver the chemotherapeutic agent paclitaxel to these tumors via HEV [65]. These MECA-79-NPs were formed from polyethylene glycol (PEG)ylated poly(lactic-co-glycolic acid) (PLGA), an organic polymer that is biodegradable. The addition of PEG to the surface of the PLGA NPs is important for evasion of the mononuclear phagocytic system and prolongation of circulatory time [66]. MECA-79-NPs containing paclitaxel were administered intravenously to humanized NOD.Cg-*Prkdc*^{scid}*IL2rg*^{tm1Wjl}/SzJ (NSG) mice implanted with PDAC. Encapsulation of paclitaxel inside MECA-79-NPs enhanced its delivery to the primary tumor site, resulting in increased apoptosis of tumor cells and decreased vascularization that led to reduced tumor size. Hence, we posit that this nanomedicine approach might be promising for ameliorating PDAC, once clinical trials are underway [65].

Our group also found that HEVs were formed not just in the primary tumor but also in metastatic lesions of murine breast cancer (in BALB/c), murine pancreatic cancer (in BALB/c), and human pancreatic cancer in NSG mice [10]. We thus generated a novel monoclonal antibody (MHA112) against PNAd; the results showed that ADCs comprising paclitaxel (Taxol) conjugated to MHA112 (MHA112-Taxol) were effectively delivered to HEV-containing tumors, tumor-draining LNs (TDLNs), and metastatic lesions. MHA112-Taxol treatment significantly reduced primary tumor size and metastatic lesions in murine breast and pancreatic cancers, as well as in human pancreatic cancer implanted in mice [10]. Therefore, we proposed that a HEV-targeted drug delivery platform in this scenario might enable the simultaneous targeted delivery of antineoplastic

drugs not only to TDLNs, but also to two other key sites: the primary tumor and distant metastatic lesions (Figure 2, Key figure) [10]. However, one issue with respect to immunotherapies is the paucity of attention given to the pharmacokinetics of payload delivery to the TDLNs and metastatic sites. In our study, we noted a significant amount of fibrosis not only in the primary cancer but also in TDLNs and metastatic sties [10]. We argue that this massive amount of fibrosis should in theory significantly reduce the pharmacokinetics of large molecules such as antibodies to these sites. As little as less than 1% of administered antibodies have been measured to reach tumor cells [67]. Arguably, HEV-targeted delivery has the potential to overcome this shortcoming.

Targeting HEVs with nanotherapeutics might also be considered a promising strategy for modifying the immune response in primary immune-mediated conditions, such as transplant rejection [62]. For instance, our group delivered MECA-79-coated polylactic acid MPs (MECA-79-MPs) containing the immunosuppressive agent tacrolimus intravenously to BALB/c heart-transplanted C57BL/6J mice, resulting in notable accumulation of the drug in heart allograft-draining LNs [68].

Key figure

Diagram of high endothelial venule (HEV)-targeted drug delivery to metastatic cancer

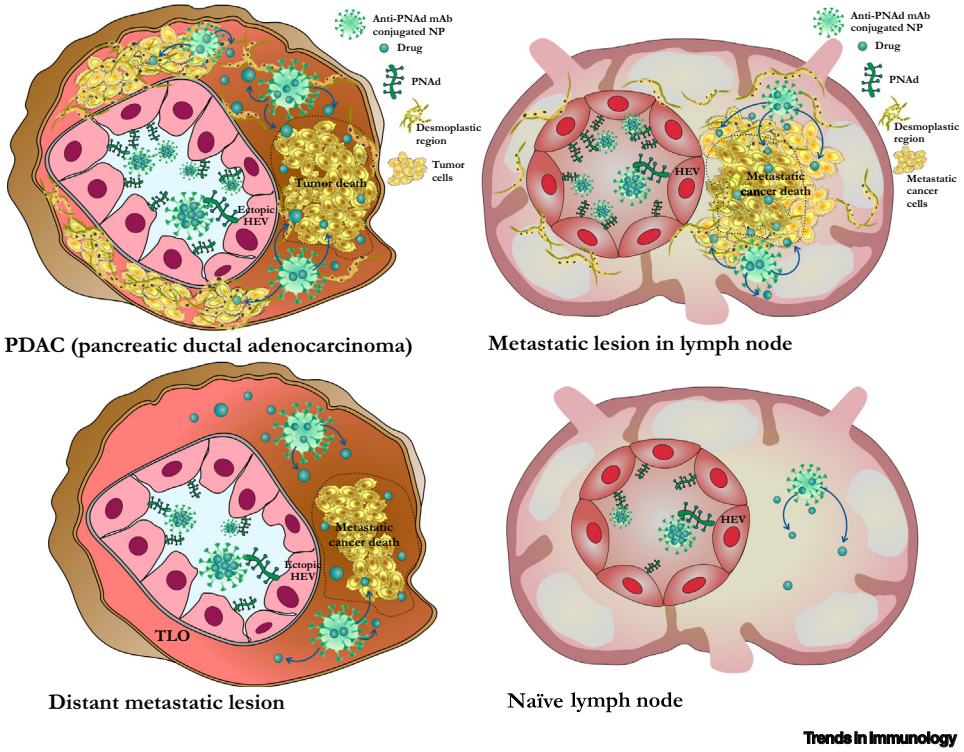

Figure 2. Drugs can be delivered simultaneously to three important treatment sites in cancers such as metastatic human pancreatic ductal adenocarcinoma (PDAC) in humanized mouse models: the primary tumor (top left), the tumor-draining lymph nodes (top right), and distant metastatic lesions (bottom left). Therapeutics can also be delivered via antibody–drug conjugates (ADCs) [10] or by encapsulation inside antibody-conjugated nanoparticles (NPs) that bind to peripheral node addressin (PNAd) on HEVs. Some off-target drug accumulation in naive lymph nodes (bottom right) and secondary lymphoid organs would also be expected, due to the presence of HEVs. Abbreviation: mAb, monoclonal antibody.

These MECA-79-MPs conjugated with tacrolimus prolonged heart allograft survival rates in the presence of lower serum tacrolimus concentrations in C57Bl/6J transplant recipients [68]. Furthermore, PEG-PLGA MECA-79-NPs loaded with an anti-CD3 T cell antibody (MECA-79-anti-CD3-NPs) targeted HEVs in the allograft-draining LNs of BALB/c heart-transplanted C57BL/6J mice [60]. MECA-79-anti-CD3-NPs accumulated more robustly in draining LNs than did nonconjugated NPs. Furthermore, treatment of cardiac allograft recipients with MECA-79-anti-CD3 NPs led to remarkably improved allograft survival compared with mice treated with free anti-CD3 antibody. Also, the regulatory T cell (Treg) populations in the allograft and draining LNs were significantly greater in mice treated with MECA-79-anti-CD3-NPs relative to the control groups, suggesting a potential mechanistic involvement for the prolonged heart allograft survival in these mice [60]. We postulate that increasing the concentrations of anti-CD3 in the allograft and draining LNs may have resulted in selective suppression of effector T cells, while sparing or promoting Tregs [69]. Anti-CD3 has been reported to promote Tregs via several mechanisms, including increased production of regulatory molecules, such as TGF-β and IL-10 [70]. Given the ectopic formation of HEVs in many autoimmune and inflammatory settings, one could argue that targeted delivery of immune therapeutics via HEVs might help to reduce the burden of these refractory conditions.

One key aspect in improving HEV-targeted delivery is to assess the trafficking pharmacokinetics of the payload to lysosomes in HECs. A significant body of research has been undertaken on cancer cells to better understand the key physical characteristics of PLGA NPs that either permit endosomal escape (thereby avoiding the acidic environment of lysosomes) or shift PLGA NP trafficking to lysosomes [71,72]. Endosomal compartments of various cell types can vary and alter the intracellular trafficking behavior of NPs. Our data suggest that a portion of PLGA NPs might bypass the lysosome and undergo exocytosis into the interstitium of the LN [60]. PLGA NPs might then be biodegraded by hydrolysis (into lactic acid and glycolic acid), and their payloads released into the interstitium of HEV-harboring tissues.

Thus, we posit that HEV-targeted nanotherapeutics may represent an encouraging and selective method for manipulating the immune response in certain scenarios such as transplantation [60], but certainly pending further and robust validation in additional preclinical models and ensuing clinical trials. Table 2 provides an overview of past studies that have demonstrated the presence of HEVs in cancers and those in which HEV positivity was utilized as a major component of treatment strategies.

Concluding remarks

HEVs are unique segments of the vasculature that have long piqued the curiosity of scientists [73]. As chief arbiters for the migration of lymphocytes to LNs, HEVs are essential for the initiation of adaptive immunity. HEVs can be differentiated from other blood vessels by their expression of PNAd glycoproteins, recognized by a group of antibodies. These antibodies have resulted in the identification of ectopically formed HEVs in organs affected by inflammatory conditions, autoimmune diseases, or cancers [10,14–22,36–41,55,64,74].

The presence and density of HEVs in many malignant lesions correlate with reduced tumor size and higher survival rate in human patients, perhaps due to greater tumor infiltration by lymphocytes, but also with worsened outcomes in some cases. Therefore, further rigorous studies are required to pinpoint the precise relationship between HEV formation and cancer progression, which is likely to vary between cancer types, stage, and immune phenotype of the patient.

Translation of these experiments to future clinical trials represents an exciting prospect that might significantly contribute to improving the treatment of patients with metastatic solid cancers,

Outstanding questions

Tertiary lymphoid organs (TLOs) are formed in organ tissues in the context of chronic inflammation defining various immune-mediated conditions. Do these structures play a proinflammatory or an anti-inflammatory role? Can these structures provide effective gateways for high endothelial venule (HEV)-targeted therapeutics in such immune-mediated disorders?

HEVs are commonly formed in close proximity to tumors ['tumor-associated (TA)-HEVs'], but the origin of the high endothelial cells (HECs) that comprise these ectopic HEVs is unknown. Do HECs arise from pluripotent cells or endothelial cells in the organ or do they arise from another site in the body (i.e., bone marrow)?

The presence of TA-HEVs near tumors and their prognostic value could be either favorable or detrimental for cancer patients, depending on the type and location of the cancer. What immunological and molecular factors determine the prognostic value of TA-HEVs?

One potential caveat of putative HEV-targeted drug therapies for immune-mediated diseases could be the accumulation of these drugs in lymph nodes (LNs) at off-target sites. Will the increased trafficking of these drugs to areas of immune activation that contain more HEVs outweigh the risks of their accumulation at off-target LNs?

Table 2. Studies of HEVs in cancer

Finding	Refs
Correlational studies	
Presence of HEVs in a variety of human solid tumors such as lung, breast, ovarian, and colon carcinomas	[42]
Positive correlation between HEVs and length of survival of patients following resection of various solid tumors such as breast, colorectal, stomach, and NSCLC	[43,79]
Positive correlation between higher density of tumor TLOs and prognosis in human patients with PDAC	[47]
Positive correlation between the presence of HEVs, and $CD3^+$ T cell and $CD20^+$ B cell infiltration, CXCL12 and CCL21 amounts, and negative correlation with CCL20 chemokine amounts in human OSCC	[48]
Positive correlation between density of cuboidal HEVs and lymphocytic infiltration, CCL19, CCL21, and CCR7 amounts; positive correlation between flat HEVs and tumor remission in human cutaneous malignant melanoma	[49]
Presence of HEVs in mouse 4T1 breast cancer and Panc02 pancreatic cancer as well as human PDAC, respective draining LNs, and metastatic lesions in wild-type and humanized mice	[10]
Correlation between TLOs and carcinogenesis in *N*-nitrosodiethylamine-induced hepatocellular carcinoma in transgenic $IKK\beta(EE)^{hep}$ mice	[51]
Correlation between TLO and MALT lymphoma and gastric carcinogenesis in *Helicobacter pylori*-infected humans and mice	[52,53]
HEV is an important prognostic marker for relapse-free survival and overall survival in human gastric cancer patients	[54]
Interventional studies	
Anti-VEGFR2 and anti-PD-L1 antibodies induced the formation of HEVs in PyMT-expressing breast cancer lesions and Rip1-Tag2 pancreatic neuroendocrine tumors in transgenic RT2-PNET mice	[46]
Treatment of GBM with CGKRK–LIGHT increased HEV induction and subsequent cytotoxic T cell infiltration, suppressing tumor growth in C57BL/6 mice	[64]
Treatment of PDAC with MECA-79-NPs encapsulating paclitaxel led to reduced tumor size in humanized mice	[65]
Treatment of murine breast cancer, murine pancreatic cancer, and human pancreatic cancer implanted in wild-type and humanized mice with MHA112-paclitaxel ADC significantly reduced primary tumor size and metastatic lesions	[10]
LTβR-independent signaling induced HEV-like vasculature in B16-OVA tumors in mice; naive T cells entered tumors through HEV-like vasculature, enhancing antitumor immunity	[80]
Tumor-associated tertiary lymphoid structures/TLOs with $PNAd^+$ vasculature and B cell entry in immune checkpoint blockade responders were associated with increased survival; unique roles for TLS and B cells in antitumor immunity were demonstrated in human melanoma, sarcoma, and metastatic renal cell carcinoma patients	[56–58]
Treating human metastatic melanoma patients with combinatorial immune checkpoint blockade therapy with anti-PD-1/anti-CTLA-4 antibodies resulted in improved survival; mechanism of CTLA-4 blockade could depend on the production of TA-HEVs	[55]

although numerous unanswered questions remain (see Outstanding questions). An important concern regarding HEV-targeted delivery is the fact that PNAd is constitutively expressed by HEVs in virtually all LNs. Therefore, the platform could lead to the delivery of immunosuppressants or immunostimulants to all LNs, potentially leading to complications from generalized immunosuppression (e.g., viral infection) or unmitigated immune activation (e.g., cytokine storm). However, the main premise of this delivery method is to significantly reduce systemic dosing, potentially limiting the toxicity associated with this route of delivery. Furthermore, there is a common misconception that just a few draining LNs exist (e.g., in the case of cancer). However, in many patients, especially those with advanced cancers, the mass of draining LNs can be significantly larger than we can assess with standard imaging tools. Therefore, treating a larger number of LNs instead of just targeting a few might be advantageous. Last, at least in the early phases,

HEVs expand and blood flow to the draining LNs increases; therefore, the LNs that are more active might presumably receive a greater proportion of systemically administered NPs [60].

We advocate for the development of delivery of therapeutics via HEVs to target primary solid tumors, TDLNs, and metastatic lesions simultaneously. Adding other applications to HEV-targeted drug delivery approaches – for instance, to reprogram the LN stroma and improve vaccine efficacy, perform nodal and distant metastatic imaging for cancer therapy, or the putative treatment of certain immune-mediated diseases – offers HEV-targeted delivery the potential to become an optimal drug delivery method. Therefore, extensive and rigorous studies are warranted to understand the clinical relevance of HEVs and ideally realize the therapeutic potential of HEV-targeted drugs.

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Declaration of interests

The authors have no interests to declare.

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