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Review

Microanatomy of lymphocyte-endothelial interactions at the high endothelial venules of lymph nodes

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Summary. Lymphocyte trafficking into lymph nodes and Peyer's patches is mediated primarily by specifically differentiated venules, called high endothelial venules (HEVs), located in the tissue parenchyma. HEVs have a unique morphology and phenotype, which enables them to interact with circulating lymphocytes efficiently. That is, the HEV endothelial cells have a tall and plump appearance, and constitutively express multiple adhesion molecules and chemokines on their surface. These molecules can interact with cognate receptors on circulating lymphocytes, thereby mediating the stepwise and sequential lymphocyte adhesion and transendothelial migration (TEM) at the HEV endothelial luminal surface. This review summarizes the fine morphological aspects of the unique HEV endothelial cells, with special reference to the spatial distribution of the adhesion molecules and chemokines that regulate lymphocyte migration.

Key words: High endothelial venule, Lymphocyte migration, Adhesion molecule, Chemokine

Introduction

High endothelial venules (HEVs) are distinctive microvascular segments located in the upstream portion of the post-capillary venules of lymph nodes (LNs) and Peyer's patches (PPs) (Marchesi and Gowans, 1964). HEVs are generally found in the paracortical and interfollicular regions of the LN and PP parenchyma together with other blood vessels. They are also occasionally observed in the medullary cords. Morphologically, HEVs can be readily distinguished from other vascular structures, by the appearance of their 'cuboidal' to 'cylindrical' endothelial cells, which are surrounded by an unusually thick basal lamina (Wenk et al., 1974). A remarkable feature of these venules is that numerous lymphocytes are observed in their lumen, some of which are not only attached to the luminal surface, but are also embedded within the high walls of the endothelium (Claesson et al., 1971; Wenk et al., 1974; van Deurs et al., 1975).

Gowans and Knight (1964) established that the lymphocyte migration across HEVs is a physiologic process. They discovered by tracing the fate of adoptively transferred, radiolabeled lymphocytes that circulating lymphocytes continuously traffic from the blood to the lymphoid tissues, returning to the blood after passing through the thoracic duct (Gowans and Knight, 1964). They also found that the main path taken by lymphocytes from the blood to the lymph is through the walls of the specific postcapillary venules of lymph nodes (Marchesi and Gowans, 1964), which are now called HEVs. The pioneering studies by Gowans and his colleagues catalyzed subsequent studies on the mechanisms underlying the cellular interactions between migrating lymphocytes and the HEV endothelial cells, which led to the hypothesis that the interaction involves the specific recognition and interaction of molecules on the surface of lymphocytes with complementary molecules expressed on the HEV endothelial cells. It is now widely accepted that HEVs mediate the trafficking of circulating lymphocytes into the LNs and PPs by expressing combinations of adhesion molecules and chemokines in a tissue-specific manner. It is also generally accepted that lymphocyte trafficking across HEVs plays a crucial role in the maintenance of immunological homeostasis in the body by providing a mechanism for the systemic spreading of the

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immunological repertoire and by recruiting effector and regulatory cells to local sites of action, i.e. to LNs responding to an antigen (Butcher and Picker, 1996; von Andrian and Mempel, 2003; Miyasaka and Tanaka, 2004).

Over the past decade, a variety of molecules associated with lymphocyte-HEV interactions have been cloned, and their functions have been studied extensively (Campbell and Butcher, 2000; Miyasaka and Tanaka, 2004; Tanaka et al., 2004). However, less research has focused on their precise localizations or their spatial and temporal changes during the course of lymphocyte extravasation through HEVs. Although the real-time in vivo imaging of lymphocyte interactions with HEVs is now feasible, it is still performed only at the cellular level (Bajenoff and Germain, 2007), and does not allow detailed analyses of molecular interactions at the cell surface. In addition, although lymphocyte-HEV interactions can be analyzed *in vitro*, the duration of studies is limited because cultured HEV endothelial cells lose their unique morphologic and phenotypic properties within 24 to 48 hours.

It can be argued that the morphological images obtained by conventional histological methods only represent static 'snapshots' and provide insufficient information for analyzing the dynamic cellular interactions occurring *in vivo*. Nevertheless, we believe that careful studies on the spatial and temporal distribution of trafficking-associated molecules at the lymphocyte-HEV interface by conventional imaging methods can still provide useful information. Combined with state-of-the-art technologies such as two-photon laser microscopy, these methods permit an accurate understanding of the unique interactions between lymphocytes and HEVs. Bearing this in mind, here we describe in detail the morphological features of HEV endothelial cells with an emphasis on the topographical changes in cell-adhesion molecules and chemokines that occur during the course of lymphocyte trafficking across HEVs.

Morphological characteristics of HEV endothelial cells

Studies of the HEV luminal surface by scanning electron microscopy (EM) performed in the 1970s identified a distinct cobblestone-like configuration, with deep crevices between adjacent endothelial cells (Anderson and Anderson, 1976). This irregular surface relief is thought to effect a turbulent blood flow, which promotes frequent collisions between circulating leukocytes and the HEV surface (Cho and De Bruyn, 1986). Various types of cellular processes are observed on the luminal surface of HEVs (Anderson and Anderson, 1976; Umetani, 1977; He, 1985; Sasaki et al., 1994; Belz, 1998). In particular, lamellipodia-like and microvillous protrusions with diverse shapes are abundant along the junctional borders of many of the HEV endothelial cells (Fig. 1), indicating the dynamic nature of the intercellular junctions of a substantial proportion of these cells. This observation also indicates the morphological heterogeneity of HEV endothelial cells in situ, although the possibility that fixation was in part responsible for the structural variation of the surface relief of individual HEV cells cannot be completely excluded.

The subcellular components of HEV endothelial cells have been examined in detail by transmission EM. A notable feature is that the cytoplasm contains multiple subcellular organelles that reflect high metabolic activity, which are rarely observed in flat endothelial cells (Freemont and Jones, 1983). The Golgi apparatus is well-developed, and numerous small transport vesicles are observed in the apical cytoplasm (Claesson et al., 1971; Wenk et al., 1974; Anderson and Anderson, 1976; Ohmann, 1980; Andrews et al., 1982). Multivesicular bodies, mitochondria, and free ribosomes are also abundant, although Weibel-Palade bodies, a structural marker of endothelial cells, are not consistently observed in HEVs (Bailey and Weiss, 1975; Perry et al., 1992). These multiple subcellular components occupy substantial subcellular space in the HEV cells, contributing to their plump morphology and to the high wall of this peculiar endothelium (Kittas and Henry, 1979).

Cell adhesion molecules expressed on HEV endothelial cells

Under physiological conditions, circulating lymphocytes continuously adhere to the surface of HEV endothelial cells, by a stepwise and sequential process consisting of rolling, tethering, activation, arrest, and transendothelial migration (TEM) (Girard and Springer, 1995; Butcher and Picker, 1996; von Andrian and Mempel, 2003; Miyasaka and Tanaka, 2004). It is now widely accepted that each step is dependent on the mutual interaction of various cell adhesion molecules belonging to the selectin, integrin, and sialomucin families, and the immunoglobulin superfamily, expressed on the surface of lymphocytes and HEV endothelial cells (Lawrence et al., 1995; Dwir et al., 1998; Faveeuw et al., 2000; Shamri et al., 2005; Umemoto et al., 2006). As mentioned above, the surface of HEV endothelial cells and lymphocytes appears complex and dynamic; therefore, precise topographical information about these molecules is essential for a thorough understanding of the process of lymphocyte migration through the HEV endothelium.

Localization of peripheral node addressins

Peripheral node addressin (PNAd) refers to a set of multiple adhesion molecules that are expressed specifically in HEVs and interact with L-selectin. PNAd includes GlyCAM-1 (Lasky et al., 1992), CD34 (Baumhueter et al., 1993), podocalyxin-like protein (Sassetti et al., 1998), endoglycan (Fieger et al., 2003),

endomucin (Samulowitz et al., 2002; Kanda et al., 2004), and nepmucin (Umemoto et al., 2006). The protein backbone of PNAd is modified with tissue-specific O-linked oligosaccharide 6-sulfo sLeX, which collectively acts as the ligand for L-selectin (CD62L) and mediates the rolling and tethering of lymphocytes on the HEV endothelium (Rosen, 1999). The localization of PNAd on HEVs is demonstrated by the monoclonal antibody MECA-79, which produces intense cytoplasmic staining as well as luminal and abluminal reactivities (Streeter et al., 1988b).

The broad distribution of PNAd along the HEV lumen is well suited for the frequent tethering and rolling of lymphocytes observed along the entire luminal surface of HEVs, both of which are mediated by the interaction between L-selectin, expressed selectively on the tip of the microvillous processes of lymphocytes (Hasslen et al., 1995; Tohya and Kimura, 1998), and PNAd, expressed by the HEV endothelial cells (Streeter et al., 1988b). In addition, PNAd often accumulates at high levels on microvillous processes of HEV cells, near their intercellular borders (Girard et al., 1999), which may contribute to the preferential localization of the majority of adhering and transmigrating lymphocytes to the cell borders of HEVs (Yamaguchi and Schoefl, 1983b). The selective positioning of lymphocytes at this intercellular region of HEV cells is likely to be advantageous for their subsequent TEM.

In a study using colloidal gold-conjugated L-selectin-IgG, Kikuta and Rosen (1994) reported that L-selectin ligands are selectively localized to the Golgi apparatus and some vesicular structures of HEV cells, indicating these organelles as sites of L-selectin ligand biosynthesis and intracellular transport (Kikuta and

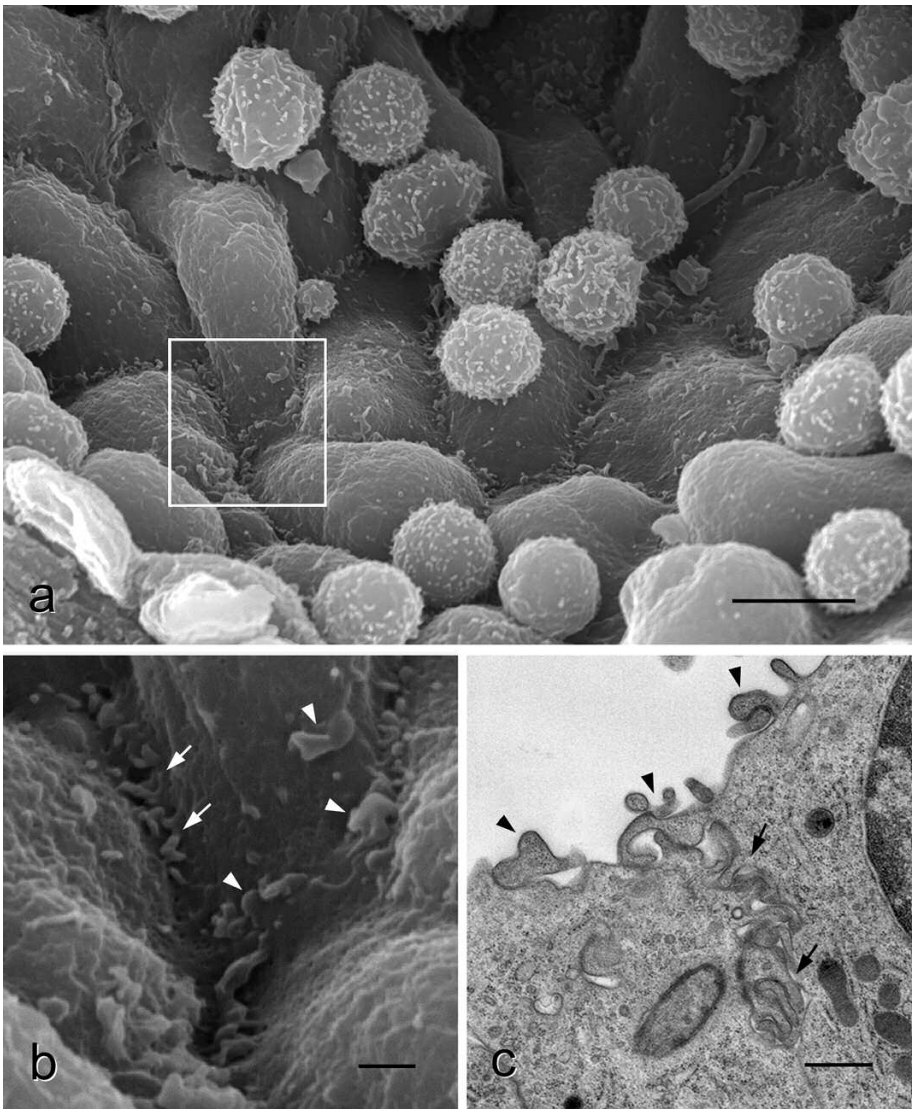

Fig. 1. a. Scanning electron micrograph of the HEV luminal surface in mouse LNs. Many lymphocytes lying on the convex endothelial cells are seen. **b.** Higher magnification of the boxed area shown in **a**. Irregularly shaped lamellipodia (arrowheads) and microvillous processes (arrows) are seen along the cell borders. **c.** Transmission electron micrograph of the HEV endothelial protrusions of various shapes, which are observed on the luminal surface (arrowheads) and between adjacent cells (arrows). Bars: a, 5 μm; b, c, 1 μm.

Rosen, 1994). We found by immuno-EM analysis that the reactive site of MECA-79 in the cytoplasm of HEV endothelial cells was not only the Golgi apparatus (Fig. 2a) and vesicles, but also included a subset of electron-dense granular structures (Fig. 2b). The exocytotic fusion of MECA-79-positive granules at the luminal surface was occasionally observed in the same specimen, indicating the existence of active transport pathways for L-selectin ligands to the HEV luminal surface (Fig. 2c). In this regard, it is of note that one PNAd component, GlyCAM-1, is shed from HEV cells and is present at readily detectable levels in the systemic circulation (Hoke et al., 1995). Given that this GlyCAM-1 is likely to be present at a very high concentration on the luminal surface HEVs, it is tempting to speculate that it plays a regulatory role in local lymphocyte-HEV interactions.

Localization of intercellular adhesion molecule-1

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin superfamily and acts as an endothelial receptor for leukocyte function antigen-1 (LFA-1), expressed on leukocytes (Marlin and Springer, 1987). Upon ligation, this endothelial adhesion molecule transmits a signal to activate Rho-like GTPases and actin polymerization (Adamson et al., 1999; Wojciak-Stothard et al., 1999), which are essential for inducing the firm adhesion and subsequent transmigration of leukocytes at the endothelial lining. In the HEV endothelium, ICAM-1 is constitutively expressed on the luminal surface and plays a key role in the firm adhesion of integrin-activated lymphocytes (Marlin and Springer, 1987; Tamatani and Miyasaka, 1990; Lawrence et al., 1995). In immuno-EM observations, ICAM-1 shows a preferential distribution to the luminal and lateral surfaces of the high endothelium, rather than to the abluminal surface supported by the basal lamina (Brown et al., 1993; Tanaka et al., 1994). Like PNAd, ICAM-1 is often concentrated on the microvillous processes of the HEV endothelial cells (Perry et al., 1992).

Recently, ICAM-1 was reported to show dynamic behavior in TNFα-stimulated human umbilical vein endothelial cells (HUVECs) during leukocyte TEM. To perform real-time imaging of TEM, Carman and Springer (2004) established a novel in vitro model in which they treated a TNFα-stimulated HUVEC monolayer briefly with CCL2/MCP-1 (for monocyte TEM), PAF (for neutrophil TEM), or CXCL12/SDF-1 (for lymphocyte TEM), removed the unbound chemoattractant, and then added the relevant leukocyte subset to the HUVEC monolayer. They found that all three classes of leukocytes showed TEM and that 5 to 10% of them clearly migrated through individual endothelial cells (transcellular route), with the remainder passing either between them (paracellular route), or passing too close to the junction for an unambiguous determination of the route. Interestingly, both the para- and transcellular diapedeses were highly associated with the presence of endothelial cup-like structures enriched

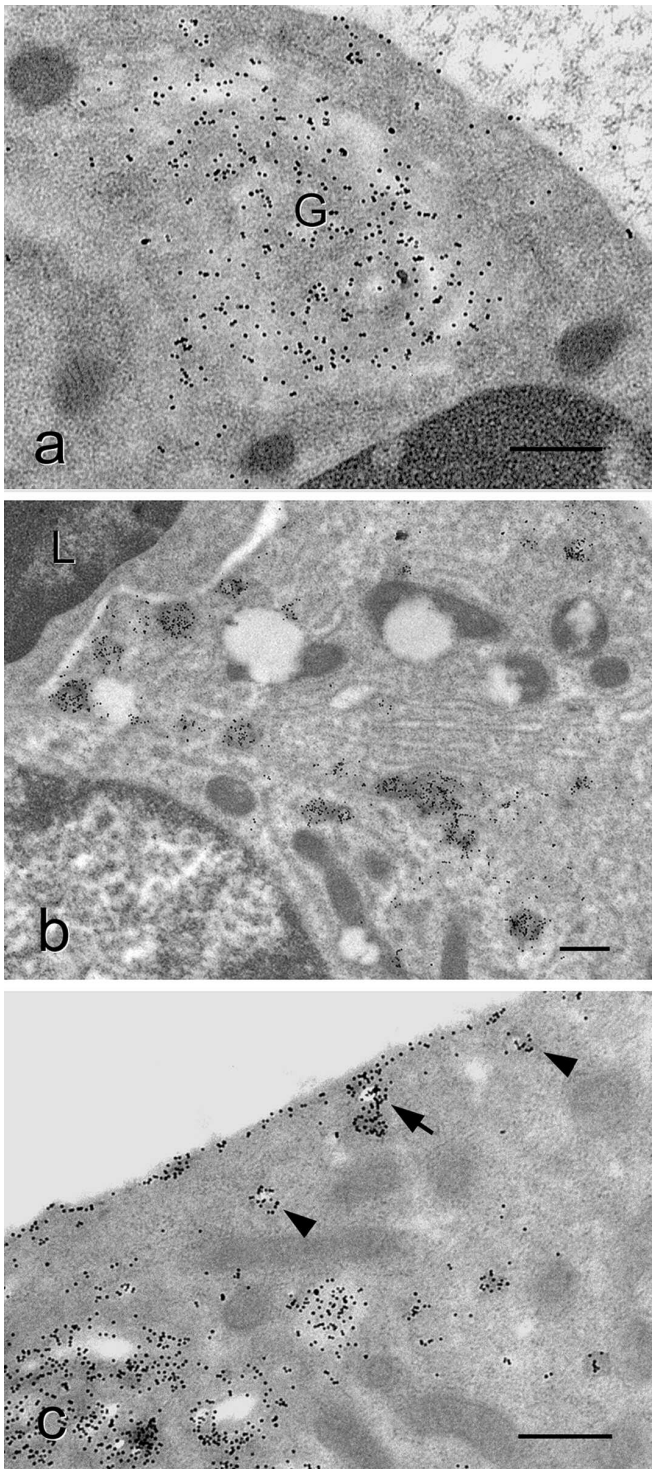

Fig. 2. Localization of PNAd (MECA-79 antigen) in mouse HEV cells detected by the post-embedding immunogold staining method. **a.** Reactive particles are concentrated in the Golgi area (G). **b.** Positive signals are also observed in electron-dense granules. **c.** MECA-79-positive granules are also accumulated near the luminal surface (arrowheads). Arrow indicates the exocytotic fusion of one of these granules. L: lymphocyte, Bars: a, 1 μm; b, c, 0.5 μm.

in ICAM-1 and VCAM-1 that surrounded the site of passage. They named this architecture the "transmigratory cup" and, given that this dynamic structure was aligned perpendicular to the endothelium and parallel to the direction of TEM, they proposed it might provide leukocytes with a physical basis for their oriented migration, i.e., from the apical to the basal side of the endothelium (Carman and Springer, 2004).

Consistent with this, Shaw et al. (2004) reported the redistribution of ICAM-1 into ringlike structures, to which neutrophil LFA-1 was also rapidly redistributed during neutrophil TEM across the TNFα-stimulated HUVEC monolayer. This LFA-1- and ICAM-1-colocalized cluster remained around the neutrophil as it transmigrated (Carman and Springer, 2004; Shaw et al., 2004). Millan et al. (2006) showed, also using TNFα-stimulated HUVECs, that endothelial ICAM-1 translocated to F-actin- and caveolin-rich regions close to the cell-cell borders and was subsequently transcytosed via caveolae upon antibody ligation, indicating that a redistribution of ICAM-1 is initiated by receptor engagement. They also showed that human T lymphoblasts transmigrated through transcellular passages by extending pseudopodia into endothelial cells at F-actin- and caveola-enriched areas in a manner partly dependent on the caveolin-I level in the HUVECs, and suggested that the ICAM-1 translocation to caveolae itself regulates TEM (Millan et al., 2006).

Sasaki et al. (1996) analyzed the three-dimensional distribution of ICAM-1 on the HEV surface by immunoscanning EM, and reported that it is extensively distributed over the luminal surface, which undulated to form microfolds and shallow microfurrows. ICAM-1 tended to be highly expressed along the sides of the folds, between which the cytoplasmic processes of lymphocytes were often extended, and in direct contact with the endothelial cells (Sasaki et al., 1996). On the lymphocytes that were apposed to HEV endothelial cells, Tohya and Kimura (1998) observed that LFA-1, the lymphocyte receptor for ICAM-1, showed capping and clustering patterns selectively at the adherent border, indicating that LFA-1's topology is also dynamically regulated during the course of lymphocyte-HEV interactions (Tohya and Kimura, 1998). Given that the cross-linking of ICAM-1 induces calcium signaling and rearrangement of the cytoskeleton (Etienne-Manneville et al., 2000), the relocation of LFA-1 and ICAM-1 on the lymphocyte and endothelial membrane, respectively, is likely to facilitate the formation of a stable adhesive structure, which may function as a scaffold for the subsequent lymphocyte TEM (van Buul et al., 2007).

It has been suggested that the intraluminal crawling of leukocytes is another indispensable step for the induction of TEM (Schenkel et al., 2004; Ley et al., 2007), and that such leukocyte locomotion is mediated at least in part by the interaction of LFA-1 and ICAM-1 on the endothelial surface (Phillipson et al., 2006). Shulman et al. (2009) carefully investigated under flow conditions the fine structure of the crawling lymphocytes on TNFα-stimulated HUVECs that had been overlaid with CXCL12, and found that the high-affinity form of LFA-1 was expressed transiently at numerous submicron focal dots in the leading edge of transmigrating chemokine-activated T cells; these LFA-1 dots were in direct contact with endothelial ICAM-1.

These high-affinity LFA-1-enriched filopodia were preferentially found underneath crawling T cells, and T cells lacking such filopodia showed no adhesion on ICAM-1 (Shulman et al., 2009). Based on functional and morphological analyses, the authors proposed that T cells crawl in a millipede-like manner on ICAM-1-expressing endothelial cells using their LFA-1-dependent filopodia, which are induced by shear forces, and that via this millipede-like locomotion, T cells scan the endothelial cells for potential TEM sites. Although these observations are provocative and interesting, scanning EM studies clearly indicate that the great majority of lymphocytes in the adhesion step or the early step of TEM in HEVs retain a relatively spherical cell body rather than the elongated shape that would be required for millipede-like movement (Fig. 3a). Adherent cells also do not show numerous filopodia on their cell body when in the lumen of HEVs (Fig. 3b), unlike the chemokine-stimulated T cells attached to ICAM-1 in vitro (Shulman et al., 2009). Thus, the mechanism of lymphocyte adhesion and TEM at HEVs still requires further investigation.

HEV endothelial cells usually contain numerous endocytic vesicular structures and are able to take up luminal tracers actively (Mikata and Niki, 1971; Wenk et al., 1974; van Deurs, 1978). Whether this ability of HEV endothelial cells is relevant to the fact that they can take up lymphocytes via transcellular or paracellular TEM remains unclear.

Localization of mucosal addressin cell adhesion molecule-1

Mucosal addressin cell adhesion molecule-1 (MAdCAM-1), a sialomucin-type cell-adhesion molecule with three (rodent) or two (human) immunoglobulin domains, was first described as a tissue-specific vascular addressin on HEV endothelial cells of the PPs and mesenteric LNs (Streeter et al., 1988a). Using the monoclonal antibodies MECA-367 and MECA-89, MAdCAM-1 was shown to be expressed exclusively on the luminal surface of the endothelium of these lymphoid tissues.

In mucosal HEVs, MAdCAM-1 is broadly distributed in the lumen, similar to PNAd, and plays an obligatory role in lymphocyte rolling and tethering by interacting with microvillus-associated α4ß7 integrin on lymphocytes (Berlin et al., 1995). The first Ig domain of MAdCAM-1 directly interacts with α4ß7 integrin. In addition, when modified with the appropriate sugar chains, the mucin-like domain of MAdCAM-1 supports L-selectin-dependent lymphocyte rolling in mesenteric LNs (Streeter et al., 1988a; Berg et al., 1993). Oshima et

al. (2001) reported that a mouse HEV-derived cell line, SVEC4-10, showed increased MAdCAM-1 expression with a strong granular staining pattern along the cell junctional border, upon stimulation with TNF-α (Oshima et al., 2001), indicating that the MAdCAM-1 expression can change upon stimulation. Given that PNAd and ICAM-1 also change their distribution upon interacting with their ligand, as described above, MAdCAM-1 may similarly relocate itself e.g., to the microvillous processes at the cell border, by interacting with a lymphocyte ligand. This possibility requires further study in vivo.

It is of note that MAdCAM-1 is prenatally expressed in mice in the lymph node anlage, and recruits lymphoid-tissue inducer cells expressing CD4, but not CD3, into the tissue (Mebius et al., 1998). During development, MAdCAM-1 expression is induced by LTα alone (Cuff et al., 1999), whereas PNAd requires LTαß (Drayton et al., 2003). At birth, MAdCAM-1 is expressed ubiquitously in all lymph node HEVs. It is replaced in the peripheral lymph nodes by PNAd within the first several postnatal days. After this time, MAdCAM-1 is expressed only in the HEVs of mesenteric LNs and PPs. In adult mice, MAdCAM-1 is also expressed in the splenic sinus-lining cells (Kraal et al., 1995), endothelial cells of the lamina propria of the small intestine (Streeter et al., 1988a), mammary gland (Tanneau et al., 1999), and epithelial cells of the choroid plexus (Wolburg et al., 1999).

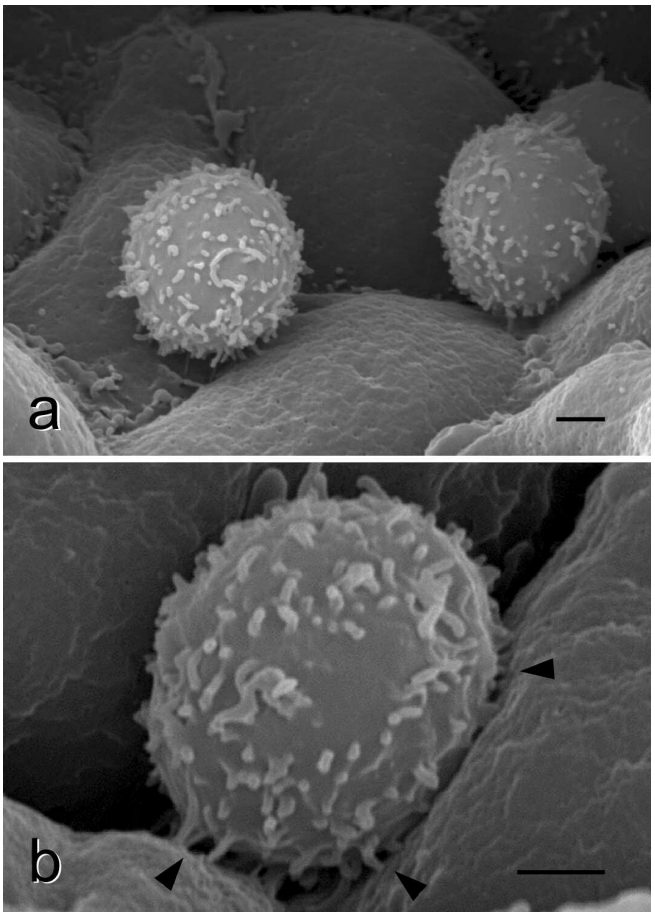

Fig. 3. a. Two lymphocytes in the early adhesion step retain their spherical shape on the HEV surface. **b.** A lymphocyte attaches to the HEV surface by short microvillous processes (arrowheads) but not by filopodia. Bars: 1 μm.

Localization of CD31

CD31 (also called platelet/endothelial cell adhesion molecule-1; PECAM-1) belongs to the immunoglobulin superfamily of cell-adhesion molecules and is selectively expressed on endothelial cells, platelets, and certain leukocyte subsets (Albelda et al., 1990; Newman et al., 1990; Watt et al., 1995). CD31 mediates both homotypic and heterotypic cell adhesion through different domains, and plays a key role in a number of important biological processes, including vasculogenesis (Cao et al., 2002) and leukocyte-endothelial adhesion during inflammation (Woodfin et al., 2007). Previously, the general consensus was that CD31 is concentrated at the lateral membrane of endothelial cells, where intercellular junctions are formed (Muller et al., 1993; Ayalon et al., 1994). In 1997, Scholz and Schaper challenged this hypothesis, by showing that CD31 is uniformly expressed on blood vessels of the myocardium (Scholz and Schaper, 1997). This finding was ratified by Feng et al. who used a pre-embedding immunogold EM procedure with multiple antibodies that reacted with different epitopes (Feng et al., 2004), and the growing consensus is that CD31 is uniformly distributed on the endothelial cells of some tissues, but is concentrated at the lateral membrane in other tissues or under specific conditions.

In the HEV endothelium, CD31 is expressed not only on the luminal surface but also at cell-cell borders, together with junctional molecules, such as VE-cadherin and junctional adhesion molecule (JAM)-A, -B, and -C (Pfeiffer et al., 2008). We confirmed this observation and further found by immuno-EM that CD31 accumulates on the microvillous processes of HEV endothelial cells at the lateral cell membrane, and can be found intracellularly, inside vesicle-like structures close to the lateral membrane (Jin et al., 2008). Prior to this, Mamdouh et al. observed that CD31 accumulates in a vesicle-like compartment connected to the cell surface, termed the lateral border recycling compartment (LBRC) in HUVECs (Mamdouh et al., 2003). They also found that CD31 is constitutively recycled to the site of leukocyte TEM by a kinesin-mediated membrane trafficking system, and proposed that the targeted recycling of CD31 from the LBRC regulates monocyte TEM (Mamdouh et al., 2003, 2008).

Given that the real-time imaging of HEVs indicates extremely dynamic membrane behaviors of the endothelial cells in situ (Bajenoff et al., 2006), the HEV endothelial cells themselves are likely to possess an

active mechanism that enables the dynamic transport of molecules and cells on and through the cell surface, and CD31's accumulation at the lateral cell membrane and in the intracellular vesicle-like structures close to the lateral membrane may depend on such a mechanism. CD31 has also been suggested to play a role in monocyte passage through the basal lamina of blood vessels by a heterophilic interaction between CD31 and a component of the extracellular matrix (Liao et al., 1995). In the case of lymphocytes, however, the gene inactivation of CD31 does not appear to affect the trafficking into LNs (Duncan et al., 1999), indicating that CD31 plays only a minor role (or a significant but redundant one) in this process.

Localization of nepmucin

Nepmucin is a recently identified Ig domain-containing sialomucin that is mainly expressed in vascular endothelial cells, including those of the HEVs of LNs but not of PPs (Umemoto et al., 2006). The nepmucin expressed in HEVs appears to mediate L-selectin-dependent lymphocyte rolling via its mucin-like domain. Nepmucin can also mediate intercellular adhesion in a heterophilic or homophilic manner via its Ig domain, by using different binding sites, and may thereby be involved in the regulation of lymphocyte TEM, first by binding lymphocytes through heterophilic adhesion to an unidentified ligand(s), and second by promoting lymphocyte TEM by a homophilic interaction between nepmucin molecules on apposing endothelial cells. Immuno-EM staining demonstrated that nepmucin is expressed on the luminal surface and on the microvillous processes located beside the intercellular junctions, and frequently accumulates at the lateral membranes of HEV endothelial cells that are loosely connected to their neighboring cells by microvillous processes. In the cytoplasm, nepmucin is found in multivesicular bodies and in tube-shaped or spherical endosomal vesicles beneath the luminal and lateral membrane, but not in the Golgi apparatus or the rER (Umemoto et al., 2006). This distribution pattern is similar to that of CD31. In cultured endothelial cells, nepmucin constitutively undergoes endocytosis more swiftly than JAM-A or VCAM-1 (Jin et al., 2008). Whether the intracellular recycling of nepmucin contributes to the process of lymphocyte TEM requires experimental verification.

Chemokines and chemokine-binding proteins expressed on or near HEVs

Accumulating evidence indicates that lymphocyte TEM through the HEV endothelium is controlled by the combinatorial expression of multiple cell adhesion molecules and chemokines, which provide navigational cues by acting sequentially on the transmigrating lymphocytes (Tanaka et al., 2004; Bromley et al., 2008). According to the current paradigm, after the selectin-dependent adhesion of lymphocytes, the activation of integrin molecules is a prerequisite for the subsequent migration steps (Foxman et al., 1997; Campbell and Butcher, 2000). In this process, certain chemokines expressed on the endothelial surface are essential for triggering integrin activation (Tanaka et al., 1993). To act locally, some of the chemokines are apparently immobilized at the endothelial surface, whereas others are associated with the HEV basal lamina and the surrounding extracellular matrix components, via chemokine-binding proteins. These binding proteins include heparan sulfate proteoglycan, DARC (Duffy antigen for receptor of chemokines) (Kashiwazaki et al., 2003), mac25/angiomodulin (Usui et al., 2002; Nagakubo et al., 2003), and type IV collagen (Yang et al., 2007). These molecules appear to be expressed concentrically, from the luminal endothelial surface to the basal lamina enwrapping the HEVs and to bind certain chemokines preferentially. For instance, DARC mainly binds proinflammatory chemokines, such as CXCL1, CXCL5, CCL2, CCL5, and CCL7 (Kashiwazaki et al., 2003), but not lymphoid chemokines, such as CCL21, CCL19, CXCL12, or CXCL13, whereas mac25/angiomodulin binds CCL21 and CXCL10 but not CCL19 (Nagakubo et al., 2003). Thus, these chemokine-binding proteins are likely to help present certain chemokines in a geographically regulated manner in and around HEVs (Miyasaka and Tanaka, 2004).

Not all endothelially presented chemokines are produced by the endothelial cells. Whereas CCL21 is produced by HEV endothelial cells in the mouse (Gunn et al., 1998; Vassileva et al., 1999; Yang et al., 2007), this does not appear to be the case in humans (Carlsen et al., 2005). Other chemokines, including CCL19 (Baekkevold et al., 2001) and CXCL13 (Ebisuno et al., 2003), are mainly produced by stromal cells in the vicinity of HEVs and appear to be delivered to the endothelial luminal surface (Rot, 2003). As described above, these chemokines bind differentially to certain chemokine-binding proteins expressed concentrically from the luminal surface to the basal lamina of HEVs. The organization of chemokines bound near the HEVs may elicit other lymphocyte responses than chemotaxis. That is, when a chemokine concentration gradient is present, lymphocytes may respond by haptotaxis, which is defined as migration along a gradient of molecules that are bound to the extracellular matrix. In the absence of such a gradient, as long as the matrix-presented chemokines are located close enough to each other in multiple rings, lymphocytes might respond by chemokinesis, which is defined as a chemoattractant-induced increased mobility of cells with random, non-directed movement (Miyasaka and Tanaka, 2004).

Localization of CCL21

CCL21 (also known as SLC, 6Ckine, TCA-4, or Exodus-2) is a CC chemokine that is constitutively

expressed by the stromal cells and HEV endothelium, and guides CCR7-positive lymphocytes and dendritic cells into the lymph node tissue parenchyma (Gunn et al., 1998). The importance of CCR7 ligand chemokines, including CCL21, for regulating T-cell trafficking into lymph nodes was clearly demonstrated in *plt*/*plt* mice, which are genetically deficient in CCL19 and CCL21 (Nakano et al., 1997). Whereas high levels of CCL21 mRNA and protein expression are present in normal mouse HEVs, CCL21 mRNA is not detectable in human HEVs, suggesting a species-specific difference in CCL21 synthesis and expression in HEV endothelial cells.

Lymphoid chemokines, including CCL21, CCL19, CXCL12, and CXCL13, are thought to be immobilized on the HEV luminal surface by negatively charged proteins, such as the broadly expressed heparan sulfate proteoglycans (Stein et al., 2000; Baekkevold et al., 2001; Phillips and Ager, 2002; Ebisuno et al., 2003), and by mac25/angiomodulin, which is expressed on the basal lamina (Usui et al., 2002; Nagakubo et al., 2003). Mac25/angiomodulin is also expressed at the microvillous processes near the cell junctions of HEVs (Girard et al., 1999). The surface localization of these lymphoid chemokines induces the activation of lymphocyte integrins and the subsequent strong adhesion of lymphocytes to the endothelium (Campbell et al., 1998; Pachynski et al., 1998).

Although previous immunohistochemical examinations indicated that CCL21 is strongly expressed in the cytoplasm of HEV cells, the only report showing a luminal localization of CCL21 was by Stein et al. (2000), who performed in vivo labeling of the HEV luminal surface by intra-arterial injection of a fluorescein-labeled anti-CCL21 antibody (Stein et al., 2000); however, the signal was only modest. In our immuno-EM analysis, the CCL21 signal was readily detectable in the Golgi apparatus and the vesicular compartment near the cell surface of HEV endothelial cells (Fig. 4), suggesting that CCL21 is produced within the cell, and then processed and transported to a vesicular compartment located close to the cell surface. However, CCL21 was rarely found on the surface of the HEV cells that expressed CCL21 in their cytoplasm, raising the possibility that CCL21 is rapidly released from the cell surface upon its immobilization and presentation. On the other hand, we found strong CCL21 expression at the basal lamina of HEVs (Yang et al., 2007), which may be involved in lymphocyte trafficking out of the HEVs and into the lymphoid tissue.

Localization of CCL19

CCL19 (also known as ELC, MIP-3ß, or Exodus-3) is another important ligand of CCR7 expressed on leukocytes, and together with CCL21, it critically contributes to the T-cell trafficking into lymph nodes through HEVs (Baekkevold et al., 2001). Although CCL19 mRNA has been detected in macrophages, dendritic cells, and some stromal cells, but not in HEV endothelial cells (Cyster, 1999), Baekkevold et al. (2001) detected CCL19 protein on the HEV endothelium using immuno-EM. They suggested that CCL19 is transcytosed from the perivascular space to the luminal surface of HEVs. Notably, however, as described for CCL21 above, CCL19 expression on the luminal surface is weak, but it is strong inside intracellular vesicles near the plasma membrane. These vesicles are of various sizes, which is consistent with vesicular transport. CCL19 is also detected in the membrane-proximal vesicles of adjacent lymphocytes, which is indicative of receptor binding (Baekkevold et al., 2001). Although DARC has been implicated in the transcytosis of IL-8 and RANTES/CCL5 (Middleton et al., 1997), CCL19 does not bind to DARC (Baekkevold et al., 2001; Kashiwazaki et al., 2003); therefore the mechanism regulating the abluminal-to-luminal transport of CCL19

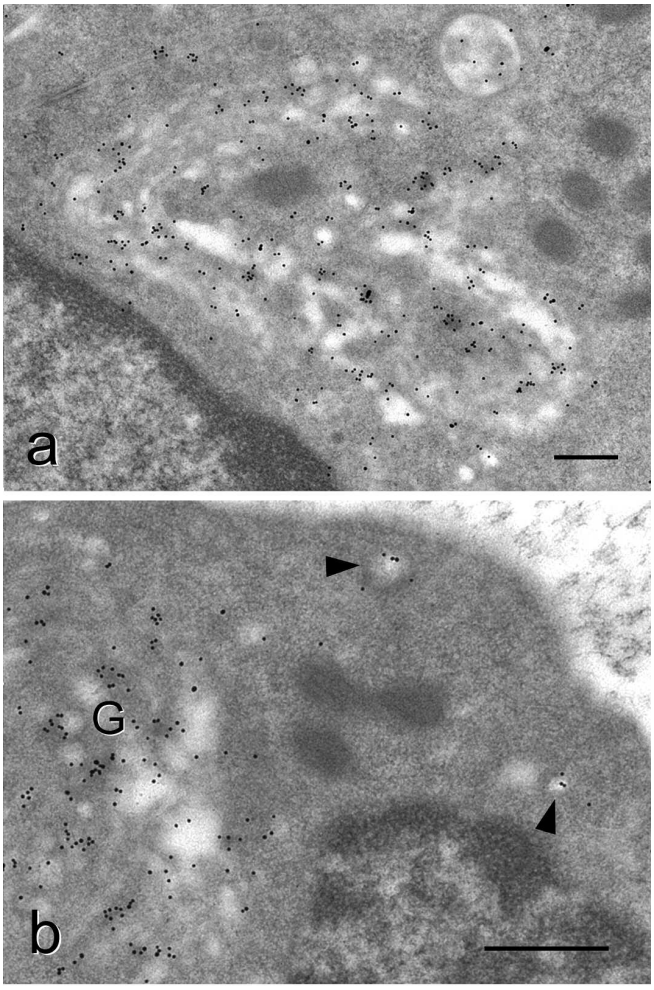

Fig. 4. Cytoplasmic localization of CCL21 in HEV cells detected by the post-embedding immunogold staining method. **a, b.** Reactive particles are concentrated in the Golgi areas (G) and in vesicular compartments near the cell surface (arrowheads). Bars: 1 μm.

remains unclear.

Other chemokines involved in lymphocyte migration via HEVs

CXCL12 (stromal cell-derived factor-1; SDF-1) and CXCL13 (B lymphocyte chemoattractant; BLC) are also known to be potent chemoattractants involved in the regulation of lymphocyte migration via HEVs (Okada et al., 2002; Phillips and Ager, 2002; Ebisuno et al., 2003). According to Okada et al. (2002), CXCL12 mRNA is not detectably expressed by HEV cells, but is expressed by cells adjacent to HEVs. However, CXCL12 protein is observed in HEV cells, indicating that CXCL12 is also transcytosed through HEV cells. Yang et al. (2007) reported that CXCL12 protein is detectable at both the luminal and abluminal aspects of HEVs, accumulating more heavily at the basal lamina (Yang et al., 2007). The CXCL12 expressed at HEVs appears to be functional, because B-cell adhesion to HEVs was disrupted when the CXCL12's receptor CXCR4 and CCR7 were desensitized (Okada et al., 2002). Consistent with this observation, CXCR4-deficient B cells migrate only poorly, if at all, into the LNs and PPs of *plt/plt* mice, which are genetically deficient in CCR7 ligand chemokines, but migrate at levels comparable to those of CXCR4-sufficient B cells into the LNs and PPs of normal mice (Okada et al., 2002). These results indicate that CXCL12 plays a redundant role with CCR7 ligand chemokines in B-cell migration across HEVs. A more recent study by Bai et al. (2009) showed that CXCL12 promotes naïve T-cell trafficking into the LNs and PPs of wild-type but not *plt/plt* recipient mice. This increased T-cell trafficking was associated with enhanced T-cell binding to HEVs and their subsequent migration into the LN parenchyma. Together with the in vitro observations, the findings of Bai et al. (2009) suggest that CXCL12 synergizes with CCR7 ligands to promote T-cell migration, by sensitizing T cells through CXCR4 signaling (Bai et al., 2009).

Discordant data have been reported concerning the expression of CXCL13 in HEVs. Although CXCL13 mRNA is not detectable at HEVs (Cyster, 1999), CXCL13 protein is detected in the HEVs inside the follicles of PPs but not in the HEVs outside the follicles (Okada et al., 2002). On the other hand, Ebisuno et al. (2003) reported that CXCL13 is expressed in the majority of HEVs in the LNs and PPs of non-immunized mice. When an anti-CXCL13 antibody was systemically administered, it bound to the surface of about 50% of the HEVs in the LNs and PPs but not to that of other blood vessels, indicating that CXCL13 is expressed at the protein level in the HEV lumen. In CXCL13-null mice, whereas B cells rarely adhered to PP HEVs, the T cells adhered normally, and superfusion of the CXCL13-null PPs with CXCL13 restored the luminal presentation of

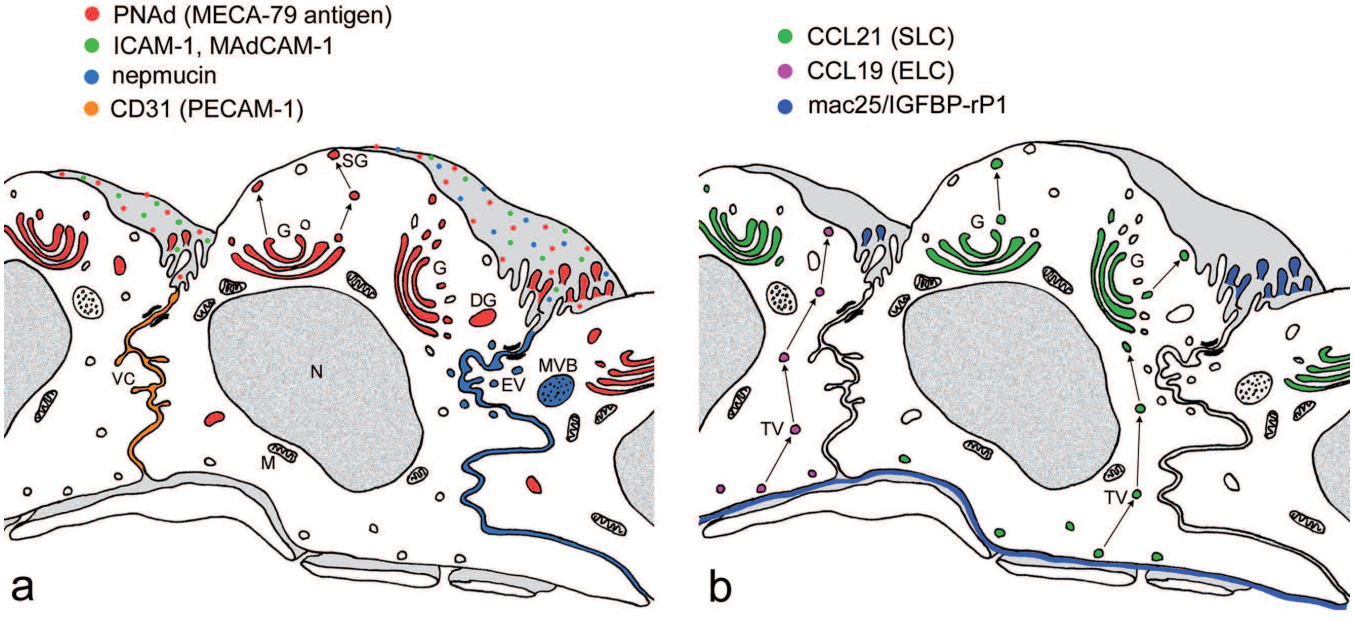

Fig. 5. Schematic drawing of the luminal and cytoplasmic localization of adhesion molecules and chemokines in HEV endothelial cells. **a.** The localization of adhesion molecules (PNAd, ICAM-1, MAdCAM-1, CD31, nepmucin) is shown. **b.** The localization of chemokines (CCL21, CCL19) and mac25/IGFBP-rP1 (a chemokine-binding molecule) is shown. DG: electron-dense granule, EV: endosomal vesicle, G: Golgi apparatus, M: mitochondria, MVB: multivesicular body, N: nucleus, SG: secretory granule, TV: transport vesicle, VC: vesicle-like compartment/structure. In both figures, arrows indicate the putative transport pathway of each molecule from the basal side toward the apical surface.

CXCL13 and B-cell arrest in PP HEVs (Ebisuno et al., 2003). These results indicate that CXCL13 plays a critical role in B-cell trafficking across PP HEVs. CXCL13 also appears to be important in LN HEVs. Although Okada et al. (2002) reported that the entry of CXCR5-deficient B cells into LNs is barely affected in normal mice, Kanemitsu et al. (2005) showed in mesenteric LNs that B cells adhered poorly to the HEVs of CXCL13-deficient mice, and that the B-cell adhesion was substantially restored when CXCL13 was added to the LNs by superfusion, as shown in PP-HEVs by Ebisuno et al. (2003). CXCL13 activates signal transduction pathways involving the small GTPase Rap1 and its effector molecule RAPL, which results in α4ß7 integrin activation in B cells (Kanemitsu et al., 2005). Thus, CXCL13 is functionally expressed in HEVs and is likely to be transported from the perivascular tissue to the apical surface, as has been observed for CCL19, CCL21, and CXCL12, although the mechanism underlying CXCL13's transcytosis remains obscure.

Migration route of lymphocytes through the HEV endothelium

The recent introduction of intravital two-photon microscopy has enabled us to visualize the dynamic behaviors of lymphocytes in lymphoid tissues under real-time conditions (Miller et al., 2002; Mempel et al., 2004; Bajenoff et al., 2006). Despite progress in the live imaging of lymphocyte migration, however, it remains unclear whether the passage of lymphocytes across the HEV wall is carried out between adjacent endothelial cells (paracellular route) or through the cytoplasm of individual endothelial cells (transcellular route) (Engelhardt and Wolburg, 2004; Vestweber, 2007). An early study by Marchesi and Gowans (1964) reported the frequent presence of intramural lymphocytes within the HEV endothelial cells and suggested that they penetrated the HEV cytoplasm prior to their passage to the extravascular space (transcellular route) (Marchesi and Gowans, 1964). Cho and De Bruyn (1986) also showed by transmission EM of serial sections of the LNs and PPs of mice, rats, and guinea pigs, that some of the lymphocytes penetrate the cell body of HEV endothelial cells and follow an intracellular path when migrating into the extravascular compartment (Cho and De Bruyn, 1986). By contrast, a study by Schoefl (1972) in which she combined electron microscopic and mathematical analyses, yielded a different conclusion, i.e., that lymphocytes use a intercellular (paracellular) path far more frequently than a transcellular one (Schoefl, 1972). The prevalent paracellular passage of lymphocytes between HEV endothelial cells was subsequently supported by a number of ultrastructural studies (Wenk et al., 1974; Anderson and Anderson, 1976; Umetani, 1977; Nishi et al., 1979; Yamaguchi and Schoefl, 1983a,b), and it is now widely accepted that the great majority of lymphocytes traverse the endothelium by following an intercellular path, although lymphocytes penetrating the cytoplasm of endothelial cells are also observed in small numbers (Yamaguchi and Schoefl, 1983b).

In contrast, in response to inflammatory stimuli, lymphocytes and other types of leukocytes appear to migrate through individual endothelial cells at substantial rates in vitro (Nieminen et al., 2006; Carman et al., 2007) and in vivo (Feng et al., 1998; Hoshi and Ushiki, 1999; Nieminen et al., 2006; Millan et al., 2006; Carman et al., 2007; Vestweber, 2007). Kvietys and Sandig (2001) suggested that in vitro paracellular passage may have resulted at least in part from the exaggerated effect of the inflammatory mediators and/or from the underdeveloped intercellular junctions between the cultured endothelial cells, as compared with the in vivo vasculature (Kvietys and Sandig, 2001). More recently, Azzali et al. (2008) reported a new transcellular pathway for lymphocyte transmigration, i.e., a canalicular pathway for B cells, by studying the mode of transendothelial migration in the HEVs of guinea pig PPs under physiological and inflammatory conditions. According to their study, B cells extravasated through the HEV endothelial cells by means of an intraendothelial canalicular formation (about 6 μm long and 2 μm in diameter) without opening interendothelial junctions, under both uninflamed and inflamed conditions (Azzali et al., 2008). Currently, it is unclear to what extent this observation applies to other lymphoid tissues and/or other animal species. Collectively, it is probably reasonable to say that lymphocytes can extravasate from HEVs using both paracellular and transcellular routes, and that the former is predominant in uncompromised HEVs in most animal species.

Concluding remarks

Over the past decade, the intrinsic importance of lymphocyte migration across HEVs has been discussed from the viewpoints of homeostatic immune surveillance and of inflammatory responses such as those observed in microbial infection (von Andrian and Mempel, 2003; Thomsen et al., 2003). We now know that the selective expression and spatial distribution of cell-adhesion molecules, chemokines, and chemokine-binding molecules in the lumen and in the vicinity of HEVs (summarized in Fig. 5) are critical for lymphocyte trafficking into lymph nodes and Peyer's patches, although overlapping but different sets of adhesion molecules and chemokines are used under uninflamed and inflamed situations. These molecules show dynamic changes in their expression and localization, at least in vitro, but we have only incomplete knowledge about the changes in these molecules during the course of lymphocyte rolling, adhesion, and transmigration across HEVs in vivo. We also need to deepen our understanding about the transendothelial pathway of lymphocytes through the HEV endothelium. The

elucidation of these issues will be critical to efforts targeting lymphocyte trafficking for therapeutic interventions.

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