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Constitutive Lymphocyte Transmigration across the Basal Lamina of High Endothelial Venules Is Regulated by the Autotaxin/Lysophosphatidic Acid Axis

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Lymphocyte extravasation from the high endothelial venules (HEVs) of lymph nodes is crucial for the maintenance of immune homeostasis, but its molecular mechanism remains largely unknown. In this article, we report that lymphocyte transmigration across the basal lamina of the HEVs is regulated, at least in part, by autotaxin (ATX) and its end-product, lysophosphatidic acid (LPA). ATX is an HEV-associated ectoenzyme that produces LPA from lysophosphatidylcholine (LPC), which is abundant in the systemic circulation. In agreement with selective expression of ATX in HEVs, LPA was constitutively and specifically detected on HEVs. In vivo, inhibition of ATX impaired the lymphocyte extravasation from HEVs, inducing lymphocyte accumulation within the endothelial cells (ECs) and sub-EC compartment; this impairment was abrogated by LPA. In vitro, both LPA and LPC induced a marked increase in the motility of HEV ECs; LPC’s effect was abrogated by ATX inhibition, whereas LPA’s effect was abrogated by ATX/LPA receptor inhibition. In an in vitro transmigration assay, ATX inhibition impaired the release of lymphocytes that had migrated underneath HEV ECs, and these defects were abrogated by LPA. This effect of LPA was dependent on myosin II activity in the HEV ECs. Collectively, these results strongly suggest that HEV-associated ATX generates LPA locally; LPA, in turn, acts on HEV ECs to increase their motility, promoting dynamic lymphocyte–HEV interactions and subsequent lymphocyte transmigration across the basal lamina of HEVs at steady state. The Journal of Immunology, 2013, 190:000–000.

Lymphocyte trafficking into secondary lymphoid organs, such as lymph nodes (LNs), is an essential homeostatic mechanism of the immune system, because it allows lymphocytes to encounter both their cognate Ag and the regulatory cells with which they must interact to initiate and maintain immune responses. The entry of naive lymphocytes into LNs occurs exclusively at the high endothelial venules (HEVs), where lymphocytes go through a multistep adhesion cascade to selectively interact with HEV endothelial cells (ECs) and, finally, to extravasate from these blood vessels. Although this multistep adhesion cascade has been studied in detail (1, 2), the precise molecular mechanism for lymphocyte egress from the HEVs (i.e., extravasation) remains unclear (3).

HEVs are readily distinguished from normal venules by their tall and plump ECs and thick surrounding basal lamina. The HEV ECs have a well-developed Golgi apparatus and numerous rough endoplasmic reticula, indicating that they have high de novo synthetic activity (4). HEVs are surrounded by multiple layers of pericyte-like cells or fibroblastic reticular cells. This fibroblastic reticular cell sheath, or perivascular sheath, creates a space outside the HEV basal lamina, termed the perivascular channel (5), through which lymphocytes move from the abluminal aspect of the HEVs to the LN parenchyma.

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Abbreviations used in this article: ALN, axillary lymph node; ATX, autotaxin; 4D, four-dimensional; EC, endothelial cell; HEV, high endothelial venule; ILN, inguinal lymph node; IMS, imaging mass spectrometry; IVM, intravital microscopy; LN, lymph node; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; MLN, mesenteric lymph node; MS/MS, tandem mass spectrometry; PLN, peripheral lymph node; ROCK, Rho-associated kinase; TEM, transmission electron microscopy.

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Autotaxin (ATX) is a secretory protein of apparently 110–125 kDa with lysophospholipase D activity (6), which hydrolyzes the abundant circulating lysophospholipid, lysophosphatidylcholine (LPC), to produce the bioactive lysophosphatidic acid (LPA). The enzymatic active site was recently determined in the catalytic domain by crystal structural studies (7, 8). We (9) and other investigators (10) previously showed that ATX is highly and constitutively expressed in the HEV ECs of LNs and Peyer’s patches. Its expression in HEVs is developmentally regulated and coincides with lymphocyte trafficking into LNs at the newborn stage (9). Kanda et al. (10) reported that i.v. injection of enzymatically inactive ATX reduced lymphocyte trafficking into the LNs, Peyer’s patches, and spleen. However, the precise mechanism by which the ATX/LPA axis regulates lymphocyte trafficking across HEVs remains largely unknown.

ATX’s product, LPA, appears to function in both autocrine and paracrine fashions to regulate the migration, proliferation, and survival of diverse cell types (6, 11). LPA transmits signals via specific receptors (LPA1–LPA5), which couple to multiple G proteins, including Gαi2/13, Gαi3, and Gq. The differential activation of these G proteins activates various intracellular signaling components, such as Rho and Rac GTPases (11). It was reported that LPA acts on T cells to induce chemokinesis (10). LPA also enhances their interaction.

In this study, we demonstrate that HEV ECs constitutively generate LPA and that, upon the inhibition of ATX alone or both ATX and LPA receptors, lymphocytes fail to migrate across the basal lamina, although they successfully infiltrate the EC layer of HEVs. LPA abrogates this inhibition, allowing lymphocytes to extravasate from HEVs again. Using in vitro and in vivo experiments, we showed that HEV-associated ATX produces LPA; LPA, in turn, acts on the HEV ECs to induce cell motility and lymphocyte release from the ECs, thereby promoting lymphocyte passage across the HEV basal lamina. Thus, the ATX/LPA axis appears to be an intrinsic regulator of lymphocyte extravasation at the HEVs.

Materials and Methods

Mice

Female C57BL/6J mice were from Japan SLC and CLEA Japan. GFP-transgenic mice (14) were a kind gift from Prof. M. Okabe (Research Institute of Microbial Diseases, Osaka University). Mice were housed at the Institute of Experimental Animal Sciences at Osaka University Medical School, and all animal experiments were performed using protocols approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine.

Reagents and Abs

HA130 was synthesized as described previously (15). Brp-LPA was from Echelon Biosciences. The 3-ccPA 18:1 (3-ccPA) and Ki61425 were from Cayman Chemical. Ki61425 and HA130 were first dissolved in DMSO and then diluted in PBS containing 0.1% (v/v) fatty acid–free BSA (PBS-BSA). Brp-LPA was directly dissolved in PBS-BSA. The 3-ccPA was supplied as a solution in chloroform, which was evaporated; the 3-ccPA was then diluted in PBS-BSA and sonicated for 5 min. These inhibitors were all freshly adjusted to the indicated concentrations for each experiment. Fatty acid–free BSA was from Sigma. LPA was from Biomedical International. 1-Myristoyl-LPC (14:0) and 1-lysoeoyl-LPC (18:2) were from Avanti Polar Lipids and Doosan Serdary Research Laboratories, respectively. Pertussis toxin and blebbistatin were from Calbiochem and Sigma, respectively. Anti-PNAd (MECA-79) mAb was purified using a size-exclusion column with size exclusion resin (TOYOPEARL TSK HW55, Tosoh) from ascites of mice inoculated with the hybridoma. Anti–PV-1 (MECA-32) and anti-ATX mAbs (9) were puriﬁed using a HiTrap Protein G column (GE Healthcare).

Transmission electron microscopy

The localization of ATX was examined by immuno-transmission electron microscopic analysis, as described previously (16) using anti-ATX mAb S9A9 (9). To analyze the effect of ATX inhibition on lymphocyte localization within HEVs, conventional transmission electron microscopy (TEM) was performed. Briefly, mice were injected in the footpad with Brp-LPA (200 μM solution). HA130 (1 μM), HA130 + LPA (200 μM), or vehicle; 45 min after the injection, mice were ﬁxed by transcardial perfusion of 2% glutaraldehyde–4% PFA solution in phosphate buffer (pH 7.4), and brachial LNs and inguinal LNs (ILNs) were harvested. After being washed, collected LNs were postﬁxed in 2% osmium tetroxide in phosphate buffer. The ﬁxed samples were then dehydrated in a graded ethanol series, inﬁltrated with propylene oxide, and embedded in Quetol 812 epoxy resin. Ultrathin sections were stained with 2% uranyl acetate and Reynolds’ lead citrate and examined using a JEM-1230 electron microscope (JEOL).

Isolation of HEV ECs from mouse LNs

PNAd+ HEV ECs were puriﬁed from pooled mouse mesenteric LNs (MLNs) and peripheral LNs (PLNs), as described (17). The purity was consistently >86%, with <2% of the cells being α-smooth muscle actin–expressing pericytes.

Measurement of ATX activity in HEV EC culture supernatants

Purified PNAd+ HEV ECs were seeded on collagen I-coated 96-well plates (BD Biosciences) (~2 × 105 cells/well) and cultured in phenol-free DMEM supplemented with 15% ATX-depleted FCS. For 4 h of incubation, the culture supernatants were collected, and ATX (lysophospholipase D) activity was assessed by the ability to release the liberated choline (9).

Imaging mass spectrometry and tandem mass spectrometry analyses

Frozen tissue sections (5-μm thick) were thaw-mounted on indium tin oxide–coated glass slides (Brewer). Ultrathin sections (70–100 nm) were serially cut sections adjacent to the ones used for the IMS analysis. To identify and detect the specific LPA signal, the ions at m/z 437, 435, 433, and 457, which include the signals of LPA (18:0), LPA (18:1), LPA (18:2), and LPA (20:4), respectively, were fragmented by collision-induced dissociation, and the product ions were analyzed. The ion signal at m/z 153 corresponded to the LPA-specific fragment ion. Tandem mass spectrometry (MS/MS) imaging analysis was performed at a raster scan pitch of 40 μm in the negative-ion-detection mode. Image reconstruction was performed using BioMap software (Novartis). For immunohistochemical analysis, the serially cut sections adjacent to the ones used for the IMS analysis were stained with Alexa Fluor 488–conjugated MECA-79 and Hoechst 33342.

Short-term lymphocyte-migration assay with FACs analysis

The migration assay with FACs analysis was performed as previously described (20). Briefly, mice were injected in the footpad with Brp-LPA (200 μM solution), 3-ccPA (250 μM), Ki61425 (200 μM), or vehicle (30 μl for front footpad, 50 μl for hind footpad); 60 min later, the recipient mice received GFP+ lymphocytes (2 × 107/mouse) and Alexa Fluor 594–conjugated MECA-32 (10% w/v). The migration assay was performed as previously described (21). In brief, mice were injected i.v. with GFP+ lymphocytes (2 × 107/mouse) and Alexa Fluor 594–conjugated MECA-32 (10% w/v).
μg/mouse). Fifteen minutes after the injection, mice were killed humanely and transcardially perfused with PBS and 4% paraformaldehyde in phosphate buffer. Collected LNs were incubated with increasing concentrations (10, 20, and 30%) of sucrose. The immunofluorescent signals were observed with an FV1000-D confocal laser-scanning microscope (Olympus). GFP+ cells in the HEV region or LN parenchyma were quantified with ImageJ software.

**Blood flow measurement at HEVs**

The mean blood flow velocity was quantitatively analyzed as described previously (22). Six HEVs were analyzed individually for each velocity measurement.

**Analysis of lymphocyte rolling and adhesion**

Mice were injected with BrP-LPA (200 μM solution), 3-ccPA (250 μM), Ki16425 (200 μM), or vehicle into the footpad (30 μl for front footpad, 50 μl for hind footpad); 60 min later, GFP+ lymphocytes (2 × 10⁷/mouse) were injected i.v. HEVs were divided into five segments (22), and the velocity of GFP+ cells rolled on, as well as the number of GFP+ cells bound to, HEV segments III-V (20–60 μm diameter) was analyzed under a fluorescence microscope (IX71; Olympus) equipped with a video recorder (DCR-TRV50; Sony).

**Two-photon intravital microscopy**

For each intravital microscopy (IVM) experiment, a single mouse was anesthetized with 2.5% isoflurane; under an operating microscope, a PE-10 polyethylene catheter was inserted into the left jugular vein for the administration of rhodamine B isothiocyanate–dextran (molecular mass 70 kDa; Sigma) and cells. BrP-LPA, 3-ccPA, or Ki16425 was injected into the right footpad of recipient mice. The right ILN was surgically exposed and adjusted for two-photon observation. Throughout the procedure, the body temperature of the mouse was maintained at 37°C using a warm plate, and the mouse was kept anesthetized under 2.5% isoflurane in air. Rhodamine B isothiocyanate–dextran (10 mg/ml, 100 μM/mouse) and GFP+ lymphocytes were injected into recipient mice via the jugular vein. The images were recorded by a Leica TCS SP5 Multiphoton Microscopy System equipped with a 20× water-immersion objective. Two-photon excitation was obtained by a Mai Tai Ti:Sapphire laser (Spectra-Physics) tuned to a wavelength of 800 nm with a FemtoControl dispersion control device (Angewandte Physik und Elektronik). Emission wavelengths of 500–550 nm (for GFP; green) and 565–605 nm (for rhodamine; red) were acquired with a nondescanned detector. For time-lapse–image acquisition, each xy plane spanned 387 × 193 μm at a resolution of 0.377 μm/pixel. Nineteen xy images, typically with a z-step spacing of 3 μm, were acquired every 30 s for 30 min and analyzed with Imaris software (Bitplane).

**Visualization of actin filaments in HEV ECs**

Freshly isolated PNAd+ HEV ECs were seeded on collagen I–coated eight-well slides (5 × 10⁵ cells/well) and allowed to adhere for 2 h in DMEM containing 20% FCS. After unbound cells were gently removed with PBS, the remaining cells were treated with Brp-LPA, 3-ccPA, HA130, or vehicle in fresh DMEM for 1 h and further stimulated with LPA or LPC (10 μM) for 1 h. After being washed, the cells were fixed in 4% PFA for 20 min, permeabilized in PBS containing 0.1% Triton X-100 for 3 min, and incubated in PBS containing 1% BSA and Alexa Fluor 488–phalloidin (1 U/ml; Life Technologies) for 1 h. The cells were then observed under a confocal microscope (Leica TCS SP5). The morphologically changed ECs were defined as cells with prominent protrusions of filopodia and/or lamellipodia.

**In vitro time-lapse imaging analysis of HEV–lymphocyte interactions**

Freshly prepared LN stromal cells and lymphocytes were seeded together on collagen I–coated eight-well Lab-Tek chambered cover glasses (Thermo Scientific) in DMEM containing 20% FCS for 4 h. After unbound cells were removed, the behavior of individual MEC-79+ HEV ECs was recorded with a phase-contrast microscope (IX71; Olympus) fitted with a CCD camera (ORCA-03G; Hamamatsu Photonics) at 1-s intervals for 30 min at 37°C in a 5% CO₂ atmosphere.

**Results**

**ATX is produced by ECs and neighboring cells of HEVs and presented on the surface of HEV ECs that regulate trafficking of blood-borne lymphocytes into the LN parenchyma**

We first examined the cell types producing ATX in LNs. As shown in Fig. 1A, ATX was found to be highly expressed in HEVs, in agreement with previous reports (9, 10). Electron microscopic analysis confirmed this observation. In addition, ATX was occasionally found in the pericytes that surround ECs (Fig. 1B).

When HEV ECs were purified and cultured in vitro, ATX activity was readily detected in their culture supernatant, as assessed by the enzyme’s ability to liberate choline from LPC (Fig. 1C). The contribution of pericytes to the production of ATX appeared to be minimal, because a contamination of α-smooth muscle actin+ pericytes was <2%. ATX activity was also detected in the culture supernatant of a stably transfected ATX-expressing EC line (MBEC4-ATX) but not in the parental MBEC4 line, indicating that ATX is constitutively produced by HEV ECs in a functionally active form.

As shown in Fig. 1D, purified MECA-79+ ECs constitutively bore ATX on their cell surface, and the binding was apparently cation independent. The exogenous addition of rATX only modestly increased the surface ATX expression levels in the purified HEV ECs (Fig. 1D). These results indicate that HEV ECs have an endogenous mechanism for binding ATX on their cell surface and that the binding sites are virtually saturated, possibly as a result of the ECs’ constitutive production of ATX. Supporting this notion, treatment of the mouse EC line MBEC4 with heparinase and heparanase, but not chondroitinase, substantially reduced ATX expression on the cell surface (data not shown). Given also that ATX can be purified by heparin agarose column chromatography (23), it is likely that ATX is immobilized on the EC surface, at least in part via specific glycosaminoglycans.

In agreement with these results, i.v. injected anti-ATX mAbs selectively bound to the luminal aspect of PNAd+ HEVs, which invariably showed the avid binding of adoptively transferred GFP+ lymphocytes; the observations were virtually identical in PLNs (Fig. 2A) and MLNs (Fig. 2B). From 15 to 120 min after lymphocyte transfer, the GFP+ cells progressively migrated away from the vessel walls to move deep into the LN parenchyma, indicating that these ATX-expressing HEVs mediated both lymphocyte binding and extravasation.

**ATX’s end-product LPA is produced locally by LN HEVs**

We next examined, using MALDI-IMS in MS/MS mode, whether ATX’s end-product LPA is locally produced in HEVs. For this to end, we first obtained, from the PLN and skeletal muscle (quadriceps femoris), the MS/MS spectra of the precursor ions at m/z 437, 435, 433, and 457, which included the specific signals of LPA (18:0), LPA (18:1), LPA (18:2), and LPA (20:4), respectively; LPA (18:2) and LPA (20:4) are the major LPA species produced by mouse rATX in vitro (24). The LPA ions were fragmented in the collision-induced dissociation cell of the mass spectrometer, and the generated signals were analyzed (Fig. 3A). The most common LPA species detected (in decreasing order of signal intensity) was LPA (18:0) >> LPA (18:1) > LPA (18:2) ~ LPA (20:4) in PLNs (data not shown). As shown in Fig. 3B, the obtained MS/MS spectra of the ion peak at m/z 437, 435, 433, and 457 indeed contained the LPA-specific fragment ion, which appeared at m/z 153. The intensity of the m/z 153 signal among the product ions of m/z 435, 433, and 457 was invariably much higher in PLNs than in the skeletal muscle, whereas nonspecific product ions (m/z 255 in m/z 435 and m/z 239 in m/z 433) were found at
comparable levels between these tissues, indicating that the signals corresponding to the LPA (18:1), LPA (18:2), and LPA (20:4) species were far more abundant in PLNs than in the skeletal muscle. In sharp contrast, in the ion spectrum at m/z 437 that includes the signal of LPA (18:0) binding to ATX only unstably (25), the LPA-specific signal (m/z 153) was found at a comparable level in these two tissues. These results are in agreement with the hypothesis that biologically relevant forms of LPA are specifically produced in HEVs.

The tissue-distribution images of LPA (18:1), LPA (18:2), and LPA (20:4) were then produced in PLN sections by visualizing the ion transition from m/z 435, 433, and 457 to 153. As shown in Fig. 3C, the signals of LPA (18:1), LPA (18:2), and LPA (20:4) were found frequently in the paracortex but only sparsely in the follicle. Notably, 52–63% of LPA signals [52.5, 63.5, and 59.5% for LPA (18:1), LPA (18:2), and LPA (20:4), respectively] were closely associated with PNAd + HEVs (within 50-μm distance from HEVs) (Fig. 3D), indicating that the particular LPA species were constitutively produced in a high proportion of HEVs. To our knowledge, this represents the first visualization of LPA in LN HEVs.

Local inhibition of the ATX/LPA axis impairs lymphocyte trafficking into regional LNs

Given the high ATX and LPA expression in HEVs, we next examined the effect of inhibiting the ATX/LPA axis in LNs. Because germline knockout of the gene encoding ATX results in embryonic death (7, 26), and conditional gene knockout animals are not available, we inhibited the ATX/LPA axis pharmacologically. We used three structurally different inhibitors of ATX and/or LPA receptor signaling: a dual inhibitor of ATX and LPA receptors, BrP-LPA [IC50 for ATX = 100–200 nM (27)]; a selective ATX inhibitor, 3-ccPA, which lacks significant agonistic activity for LPA1, LPA2, or LPA3 [IC50 = 620 nM (28)]; and an LPA receptor antagonist, Ki16425, which inhibits LPA1 and LPA3 [IC50 = 50 nM (29)].

Strikingly, the local administration of BrP-LPA induced a ∼70% reduction in lymphocyte trafficking into the draining LNs (ALNs and ILNs) but not the spleen, as assessed by the enumeration of adoptively transferred lymphocytes in each tissue 30 min after transfer (Fig. 4A). The inhibition was not obvious when BrP-LPA was administered at a suboptimal dose (10 μM; data not shown). Examination 2 h after transfer yielded similar results with less inhibition; at 3 h, the lymphocyte trafficking had returned to baseline, indicating that the inhibitory effects were transient and reversible, as expected, because small molecular weight inhibitors tend to clear rapidly from the circulation. The apparent absence of an inhibitory effect on lymphocyte trafficking to the spleen by BrP-LPA was probably due to the locally injected inhibitor not reaching the spleen at a sufficient concentration.

The ATX inhibitor 3-ccPA impaired lymphocyte trafficking into the draining LNs less potently than did BrP-LPA, and it did not inhibit trafficking to the spleen (Fig. 4A). Neither of these inhibitors affected the in vivo blood flow velocity in LNs (Fig.
administration of BrP-LPA, 3-ccPA, or Ki16425 did not affect rolling frequency, rolling velocity (Fig. 5A), or adhesion frequency (Fig. 5B). We next performed whole-mount analysis of the LNs that drained the injection site of a small molecular weight selective ATX inhibitor, HA130 \([IC_{50} = 28 \text{ nM} \,(15)]\). In the draining LNs (PLNs), HA130 induced a marked accumulation of adoptively transferred lymphocytes within or in the vicinity of the HEVs, indicating that it strongly inhibited lymphocyte extravasation into the LN parenchyma; this accumulation was not observed in nondraining LNs (MLNs) (Fig. 5C). BrP-LPA yielded similar results (data not shown). The administration of vehicle alone affected neither the lymphocyte binding to HEVs nor their migration into the LN parenchyma. Notably, the inhibitory effect of HA130 was abrogated by the s.c. injection of LPA, which, reinduced lymphocyte migration into the parenchyma. Quantification of the frequency of transmigrating cells into the LN parenchyma confirmed this observation (Fig. 5D); HA130 decreased the frequency of GFP\(^*\) cells in the parenchyma by \(\sim 50\%\) compared with the vehicle control, and the coadministration of LPA with HA130 recovered this frequency to \(\sim 80\%\) of the control level. LPA alone increased the frequency of GFP\(^*\) cells in the parenchyma by \(\sim 20\%\). No changes were observed with any of these treatments in nondraining LNs, including MLNs. These strongly support the hypothesis that the ATX/LPA axis is important for the lymphocyte extravasation at HEVs and that LPA acts downstream of ATX.

Two-photon IVM studies further verified that ATX inhibition blocked the lymphocyte extravasation from HEVs. As shown in Fig. 5E and 5F (see also Supplemental Video 1), analysis of IVM data using Imaris software allowed the visualization of adoptively transferred GFP\(^*\) cells interacting with HEVs. In control mice, the four-dimensional (4D) analysis showed that many GFP\(^*\) cells were in the act of penetrating the HEV wall, with part of the cell body remaining in the vessel lumen and part protruding into the abluminal side (Supplemental Video 1). BrP-LPA or 3-cc-PA markedly reduced the frequency of cells extravasating from the HEVs of the draining LN, whereas Ki16425 did not (Fig. 5E, 5F). As observed in the short-term homing assay (Fig. 4A), Ki16425 did not inhibit extravasation significantly.

TEM analysis strongly supported the above observations. Forty-five minutes after the footpad injection of HA130 or BrP-LPA, but not vehicle alone, prominent lymphocyte accumulation was observed within the EC and sub-EC compartments (Fig. 6) in the draining ILN. In some HEVs, the lumen became narrow; this was particularly prominent in HEVs in which lymphocytes accumulated heavily in the EC and sub-EC layers, whereas it was not observed in non-HEV–type blood vessels or in HEVs treated with vehicle alone. Virtually identical observations were made in other PLNs, such as ALNs and brachial LNs, when the inhibitor was injected into their draining areas (data not shown). These results indicate that both lymphocyte accumulation and luminal narrowing were selectively observed in the HEVs of LNs that had received an ATX inhibitor injection and that lymphocyte accumulation within the EC compartment probably led to the luminal narrowing. However, the blood flow within LNs was unaffected by these treatments, as described above, indicating that the HEV luminal narrowing did not compromise the total blood flow in the draining LNs and, thus, did not affect the initial lymphocyte delivery into the LNs. Moreover, the frequency of lymphocytes that bound to or infiltrated the EC layer did not appear compromised in

### FIGURE 2.

ATX is luminally expressed on LN HEVs, which allow lymphocyte binding and transmigration. Alexa Fluor 594–labeled MECA-79 mAb (15 \(\mu\)g), which labels HEVs, and Alexa Fluor 647–labeled anti-ATX mAb (20 \(\mu\)g) were injected i.v. into each mouse 10 min before sacrifice. GFP\(^+\) splenic cells (2 \(\times\) 10\(^7\)/mouse) were injected i.v. and examined after the indicated times. PLNs (A) and MLNs (B) were harvested and used for whole-mount analysis. Alexa Fluor 594 rat IgM and Alexa Fluor 647 rat IgG (15 \(\mu\)g) were used as negative controls for the Ab injection. A representative of three independent experiments is shown. Scale bars, 50 \(\mu\)m.
the inhibitor-treated HEVs, indicating that ATX inhibition mainly impaired lymphocyte extravasation and not lymphocyte adhesion or entry into the HEV EC layer. Notably, as observed by multi-photon IVM analysis, the TEM images showed that lymphocyte passage from the EC compartment to the surrounding LN parenchyma was promoted by s.c. injected LPA (Fig. 6), thus abrogating the inhibitory effects observed with HA130. These data strongly suggest that the ATX/LPA axis promotes lymphocyte extravasa-

FIGURE 3. LPA is detected in the vicinity of LN HEVs. (A) The detection of LPA species by MALDI-MS/MS analysis. The precursor ions at m/z 437, 435, 433, and 457, which include the signals of LPA (18:0), LPA (18:1), LPA (18:2), and LPA (20:4), respectively, were collected and fragmented by collision-induced dissociation in a mass spectrometer, and the generated signals were subsequently analyzed. The signal at m/z 153 corresponds to the LPA-specific fragment ion. (B) The MALDI-MS/MS spectra of the precursor ions at m/z 437, 435, 433, and 457 were obtained from the PLN and skeletal muscle (quadriceps femoris). These product ion spectra invariably showed the presence of LPA-specific signal (m/z 153). Some nonspecific signals (m/z 255 in m/z 435 and m/z 239 in m/z 433) were also observed. (C) Tissue-distribution image of LPA species in LN sections. The LPA (18:1)-derived signal (ion transition from m/z 435 to 153), LPA (18:2)-derived signal (m/z 433 to 153), and LPA (20:4)-derived signal (m/z 457 to 153) were overlapped with MECA-79 staining on a serial section of PLN (HEVs; green). Nuclei were counterstained with Hoechst 33342 (blue). The signals associated with HEVs are enclosed by dashed lines. Scale bars, 100 μm. (D) The proportion of LPA signals present in the indicated distance from HEVs. Data are representative of two independent experiments. F, Follicles.
The ATX/LPA axis enhances HEV EC motility and promotes dynamic lymphocyte–EC interactions in a myosin II–dependent manner

We next examined how the ATX/LPA axis affects lymphocyte passage through the HEV basal lamina. Given that LPA induced only modest chemokinesis but no chemotaxis in naive lymphocytes, at least in our hands (data not shown), we reasoned that LPA acted mainly on HEV ECs rather than on lymphocytes. To test this hypothesis, we examined the response of purified HEV ECs to LPA and LPC. As shown in Fig. 7A, both LPA and LPC induced prominent cytoskeletal changes in the ECs in agreement with previous observations (9). HA130 or BrP-LPA abrogated the LPC-induced changes; however, the LPA-mediated changes were abrogated by BrP-LPA but not HA130. These results are consistent with the hypothesis that LPC is converted to LPA by HEV-derived ATX and that LPA, in turn, acts on HEV ECs as an effector molecule to induce their motility.

We next performed in vitro lymphocyte-transmigration assays in the presence or absence of ATX/LPA axis inhibitors. As shown in Fig. 7B and Supplemental Video 2, time-lapse analysis revealed that purified HEV ECs elicited avid lymphocyte binding and transmigration; this process was highly dynamic, with bound lymphocytes migrating swiftly underneath the ECs and, once trapped there, moving out extracellularly. Notably, HA130 primarily inhibited the release of lymphocytes that had migrated underneath HEV ECs, whereas LPA abrogated this inhibitory effect to rein-duce dynamic lymphocyte interactions with the HEV ECs.

We next examined the involvement of Rho-associated kinase (ROCK)–myosin II signaling in the ATX/LPA-dependent lymphocyte–HEV EC interactions, because HEV ECs express LPA4 and LPA6 (A. Takeda and E. Umemoto; unpublished observation), which couple to the Rho/ROCK–myosin II–dependent signaling pathway (11). As shown in Fig. 7C and Supplemental Video 3, treatment of both HEV ECs and lymphocytes with a myosin II inhibitor blebbistatin, but not Gaia inhibitor pertussis toxin (data not shown), severely attenuated lymphocyte detachment from ECs. When only HEV ECs were treated with blebbistatin, HEV ECs showed reduced membrane ruffle formation (Fig. 7D, Supplemental Video 3), and lymphocytes trapped underneath the ECs failed to move out extracellularly (Fig. 7E, Supplemental Video 4), as was observed with ATX inhibition with HA130 (Fig. 7B). These data support the idea that ATX’s end-product, LPA, regulates EC motility in a myosin II–dependent, but Gaia-independent, manner, and this regulation plays an important role in the reversible adhesive/deadhesive interactions between lymphocytes and HEV ECs.

Based on the evidence presented in this article, we propose the following model for the role of the ATX/LPA axis in lymphocyte extravasation from HEVs (Fig. 8). The ATX/LPA axis is...
FIGURE 5. The ATX/LPA axis regulates lymphocyte extravasation. (A and B) Mice were injected in the footpad with BrP-LPA (200 μM solution), 3-ccPA (250 μM), Ki16425 (200 μM), or vehicle; 60 min later, GFP+ lymphocytes (2 × 10^7/mouse) were injected i.v. HEVs were divided into five segments. The frequency (left panel) and velocity (right panel) of GFP+ cells rolled on HEV segment III–V (A) and the number of GFP+ cells bound to the equivalent fragments (B) were analyzed using IVM. (C) HA130 (1 μM solution) or control solvent was injected into the footpad of mice, and 45 min later, LPA (200 μM) or control solvent was injected. GFP+ lymphocytes (2 × 10^7/mouse) and Alexa Fluor 594–conjugated MECA-32 (10 μg/mouse) were injected into the tail vein 30 min after the second injection, and the mice were sacrificed 15 min later. Whole-mount analysis was performed on PLNs and MLNs. Scale bars, 100 μm. (D) Lymphocyte migration was quantified by taking a series of photographs of these LNs (>60 photographs of each group), and the number of GFP+ cells/photograph was counted using ImageJ software. The cells within and around HEVs were counted manually, and this number was subtracted from the total cell number to obtain the cell number in the parenchyma. The abundance of HEVs in each group was comparable. The percentage of cells in the parenchyma was calculated as the ratio of the cell number in the parenchyma/total number of cells in each photograph. (E) Representative images from time-lapse movies obtained in the ILN. Blood vessels were visualized by i.v. injection of rhodamine-dextran (red), and HEVs were identified by their unique morphology. The obtained images were postanalyzed using Imaris software, which renders the optical sections into 4D images, thus allowing us to discriminate the GFP+ lymphocytes located in the abluminal side of rhodamine-dextran–filled HEVs from those localizing in the luminal side; extravasating GFP+ lymphocytes appeared green in the absence of any red signal in the background. These images are also presented in Supplemental Video 1. (F) Quantification of extravasating cells from HEVs in the ILN of mice that had been injected s.c. with vehicle alone, BrP-LPA, 3-ccPA, or Ki16425. Data in (A–E) were obtained from more than three independent experiments. Data in (F) were obtained from eight independent intravital 4D data sets for each inhibitor group. *p < 0.05, ***p < 0.001. ns, Not significant.
probably not required for lymphocyte infiltration into the EC layer but is indispensable for lymphocyte extravasation from HEVs. ATX is first secreted by HEV ECs and neighboring cells and immobilized on the EC surface via glycosaminoglycan chains, such as heparan sulfate. The EC-captured ATX converts circulating LPC to LPA in situ and LPA then bind to its cognate receptors on ECs to induce their motility and permeability, as well as lymphocyte detachment from the ECs, which may collectively drive directional lymphocyte movement from the basal aspect of HEV ECs to the surrounding LN parenchymal compartment. Finally, lymphocytes squeeze through gaps in the basal lamina using contractile force, which may be initiated by chemokines or other factors expressed on the HEV basal lamina (32).

**FIGURE 6.** The ATX/LPA axis promotes lymphocyte passage through the basal lamina. (A) Transmission electron micrographs of HEVs in the draining LNs (ILN) of mice treated with vehicle alone, BrP-LPA, HA130, or HA130 + LPA. Scale bars, 2 μm. Color was added (right panel of each image) to show the localization of lymphocytes (green) within the HEV EC layer (yellow) or perivenular channels. The basal lamina is colored blue. LNs were harvested 45 min after injection. Luminal narrowing and intra-EC lymphocyte accumulation were observed to various degrees (slight, moderate, or prominent) in animals treated with BrP-LPA or HA130 but not vehicle alone. Coadministration of LPA abrogated the luminal narrowing and intra-EC lymphocyte accumulation. (B) Total numbers of lymphocytes that accumulated in the EC layers and sub-EC compartments (lymphocytes that transmigrated across the lamina densa, but did not reach the lamina reticularis) per EC were quantified. Five to thirteen HEVs in each group were statistically analyzed. *p < 0.05.
FIGURE 7. The ATX/LPA axis enhances EC motility and promotes lymphocyte release from HEV ECs in a myosin II–dependent manner in vitro. (A) LPA- or LPC-induced morphological changes and effects of ATX/LPA inhibitors. Isolated HEV ECs were incubated with Brp-LPA, HA130, or vehicle alone and then 10 μM LPA, 10 μM LPC, or vehicle was added. The y-axis shows the ratio of morphologically changed HEV ECs/normal HEV ECs. The morphologically changed HEV ECs were defined as cells with prominent protrusions of filopodia and/or lamellipodia. More than 100 HEV ECs were counted in each group. Data are representative of two independent experiments and presented as the mean ± SD. (B) Time-lapse imaging of lymphocyte–HEV EC interactions in vitro. Images are individual frames from continuous time-lapse movies (Supplemental Video 2). LN stromal cells and lymphocytes were plated and incubated for 4 h in serum-containing medium. After nonadherent cells were gently removed, the HEV ECs (colored pale yellow and encircled by a dotted line) were identified by MECA-79 staining and recorded for 30 min (untreated, upper panel). The same HEV ECs were further monitored in the presence of HA130 (500 nM) for 30 min (middle panel) and subsequently incubated with LPA (20 μM) for 30 min (lower panel). Lymphocytes continually locomoting underneath a single HEV EC are shown in blue, whereas those in the act of ingress to and egress from the EC are shown in green and red, respectively. (C) Effects of myosin II inhibition on lymphocyte–HEV EC interactions. HEV ECs were incubated in serum-containing medium and recorded for 20 min (untreated, upper panel). The same HEV ECs were further monitored in the (Figure legend continues)
Discussion

In this study, we show that the ATX/LPA axis may play a previously unknown role in the final step of lymphocyte extravasation (i.e., migration across the HEV basal lamina). We verified that ATX is secreted by HEV ECs in an active form capable of generating LPA and that ATX is immobilized on the luminal surface of HEVs in vivo. ATX’s end-product (LPA) was detected in high proportions of HEVs in vivo. The s.c. administration of BrP-LPA, which inhibits ATX and LPA receptors at micromolar concentrations (33), strongly inhibited lymphocyte extravasation; 3-ccPA (28) and HA130 (15), both of which selectively inhibit ATX, also inhibited lymphocyte extravasation in draining LNs. LPA abrogated these inhibitory effects.

The more potent effect of BrP-LPA compared with 3cc-PA can be interpreted in at least two ways: first, BrP-LPA could be a dual inhibitor of ATX and LPA signaling, thus exhibiting a stronger effect on HEV ECs than a selective ATX inhibitor, such as 3-ccPA. Second, BrP-LPA may also act on non-HEV cells by inhibiting LPA receptors, because several other cell types in LNs express LPA receptors. Although we cannot completely exclude this possibility, the fact that another selective ATX inhibitor, HA130, a synthetic thiazolidinedione compound that inhibits ATX-mediated LPA production by binding to the catalytically active site of ATX (15), recapitulated the effects of BrP-LPA or 3-ccPA in whole-mount and TEM analyses of HEVs argues against this possibility. Although 3-ccPA is reported to be a weak agonist of LPA4 (34), this receptor is not expressed by HEV ECs (A. Takeda and E. Umemoto, unpublished observations).

Multiphoton IVM, whole-mount, and TEM analyses of LNs showed that the s.c. administration of HA130 induced marked lymphocyte accumulation within the EC and sub-EC layers of HEVs in draining LNs. These results were judged to be due mainly to HA130’s inhibition of lymphocyte passage across the HEV basal lamina, because HA130 had little effect on lymphocyte rolling, lymphocyte binding to the HEV luminal surface, or subsequent infiltration into the EC layer in vivo. HA130 also did not inhibit lymphocyte chemotaxis toward CCL21 in vitro at its predicted concentration in draining LNs after s.c. injection, excluding the possibility of HA130’s nonspecific lymphocyte inhibition.

Our finding that an antagonist of LPA1 and LPA6, Ki16425, did not affect lymphocyte extravasation in vivo was unexpected, given our previous observation that this drug effectively inhibits the motility of purified HEV ECs in vitro (9). One explanation is that HEV ECs express multiple LPA receptors, including LPA4 and LPA6, which cannot be inhibited with Ki16425 (29), and signaling via these receptors may be more critical for the regulation of lymphocyte–HEV EC interactions, although Ki16425-inhibitable signaling may also play a role in EC motility. Alternatively, because LPA1 is expressed mainly by cells closely attached to HEV ECs (possibly pericytes; A. Takeda and E. Umemoto, unpublished observations) but not by HEV ECs themselves, LPA1-expressing non-HEV EC population(s) might have been included inadvertently in the “purified” HEV EC preparation used in our previous study (9).

All of the inhibitory effects on lymphocyte trafficking in draining LNs were observed by injecting inhibitors s.c. in the current study. We initially administered these inhibitors i.v. but observed only minor effects on lymphocyte trafficking. This was probably because these compounds are cleared rapidly from the circulation once they are introduced into the blood. For instance, HA130 is a nanomolar ATX inhibitor in vitro but could only effectively reduce LPA levels in the peripheral blood for several minutes following a bolus i.v. injection (15). Therefore, we injected inhibitors into the footpad and examined their effects in the draining LNs. With this method, the injected material is gradually released from the injection site and continues to enter the draining LNs for more than an hour; however, once it enters the systemic circulation it is cleared rapidly without eliciting systemic effects. Thus, we believe that the s.c. injected inhibitors acted in LNs for a much longer time than when they were administered i.v. As discussed above, that all of these ATX inhibitors yielded similar results, even though they are structurally different, supports the idea that they elicited their effects in a specific manner.

An obvious question is “What mechanism impedes lymphocyte passage across the HEV basal lamina upon ATX/LPA inhibition?” Is it because the ATX/LPA axis provides a positive migratory signal or because it counters a negative retention signal in situ, or both? One possibility is that LPA signaling weakens adhesive interactions between the lymphocytes trapped in the EC layer and the HEV ECs, which may promote lymphocyte passage from the sub-EC compartment to the LN parenchyma. In this regard, a recent report by Mionnet et al. (35) requires attention; they showed that HEVs create “pocket” structures within which lymphocytes reside for several minutes before entering LN parenchyma. Our observations that ATX inhibition induced marked lymphocyte accumulation within the EC compartment and that such accumulation could be resolved by local administration of LPA may point to the possibility that such transient lymphocyte retention in the HEV “pocket” structures under physiological conditions is regulated, at least in part, by the ATX/LPA axis. As to the possible mechanism of weakening of cell adhesion between lymphocytes and HEV ECs, Gα12 activation, which can occur upon the ligation of LPA4 or LPA6, was shown to decrease the phosphorylation of focal adhesion kinase and paxillin, causing integrin displacement from the adhesion substratum (36). Another possibility, not mutually exclusive, is that LPA acts on ECs to increase their motility, which may also interfere with the adhesive interactions between transmigrating lymphocytes and ECs. In this regard, it is of note that LPA can induce Rho-dependent morphological changes in ECs that should enhance EC permeability (13, 37). A more recent study indicated that LPAů induces morphological changes in ECs by activating the Gα13–Rho signaling pathway (38); LPAů is present of myosin II inhibitor blebbistatin (50 µM, lower panel), (D) Effects of blebbistatin on HEV EC motility. HEV ECs were cultured in serum-containing medium and recorded for 20 min (untreated, upper panel). The same HEV ECs were further monitored in the presence of blebbistatin for 20 min (lower panel). Arrowheads indicate the membrane ruffles of HEV ECs. EC is delineated by the white dashed line. (E) Effects of myosin II inhibition in HEV ECs on lymphocyte–HEV EC interactions. LN stromal cells and lymphocytes were incubated in serum-containing medium for 4 h and treated with vehicle (DMSO, upper panel) or blebbistatin (lower panel) for 30 min. After the cells were washed, untreated GFP+ lymphocytes were added, and lymphocyte–HEV interactions were monitored. In vehicle control, several cells migrated underneath EC, and some were in the act of egress from EC (blue arrows), whereas some were in the act of ingress to the EC (red arrowheads). When HEV ECs were treated with blebbistatin (lower panel), the lymphocyte egress from the EC was affected. Lymphocytes that had been located underneath the EC (encircled by a white dashed line), as well as those newly added to the EC (encircled by a red dashed line) remained trapped underneath HEV EC and failed to move out extracellularly. (B–E) Original magnification ×1000. *p < 0.05, **p < 0.01.
expressed by HEV ECs. Further investigation is required to delineate the LPA receptor–signaling pathways and clarify the mechanism underlying the ATX/LPA-dependent lymphocyte passage from the HEV sub-EC compartment to the LN parenchyma.

Considering that LPA is a potent bioactive mediator that can specifically inhibit ATX at biologically relevant concentrations in vitro (39), it is plausible that some intrinsic mechanism(s) regulates the functional activity of the ATX/LPA axis in HEVs. In this regard, a recent study showed that newly produced LPA in the circulation is degraded by lipid phosphate phosphatases expressed by various cell types, including vascular ECs (40). In addition, the blood flow in the HEV lumen may help to avoid an excessive local accumulation of LPA.

Human naive T cells express LPA2 predominantly (41). Kanda et al. (10) also showed that human peripheral blood T cells respond to a chemokinetic action of LPA. However, the LNs of LPA2-deficient mice have normal cellularity and structure (T. Tanaka and E. Umemoto, unpublished observations). Our adoptive-transfer experiments revealed that LPA2-deficient lymphocytes tended to migrate into LNs more efficiently than did their wild-type counterparts (A. Takeda and E. Umemoto, unpublished observations), indicating that the LPA2-mediated signal plays a relatively minor role in lymphocytes in the context of transmigration across HEVs or that LPA receptors other than LPA2 are important for lymphocytes. In addition, naive mouse lymphocytes responded only modestly to LPA by chemokinesis but not chemotaxis at least in our hands, as described above. Therefore, we speculate that the ATX-derived LPA acts mainly on HEV by chemokinesis but not chemotaxis at least in our hands, as described above. Therefore, we speculate that the ATX-derived LPA acts mainly on HEVs, although we do not rule out the possibility that LPA acts on lymphocytes as well. While the present manuscript was being revised, Zhang et al. (42) reported that naive mouse T cells possess Mn^2+-activatable receptors for ATX on their surface. They also found that lymphocytes pretreated with exogenous ATX efficiently transmigrate across an EC monolayer in the presence of LPC, at least in vitro. It was also shown that inhibition of ROCK–myosin II, a downstream component of LPA signaling, results in impairment of lymphocyte transmigration across HEVs in vivo (43). Thus, additional studies are required to determine whether the control of lymphocyte extravasation from HEVs occurs solely through EC-intrinsic signaling, signaling in lymphocytes, or both. Given that pericytes physically associated with HEVs also express ATX, the role of pericyte-derived ATX in the regulation of lymphocyte extravasation is another subject of future investigation.

Collectively, our data strongly suggest that the ATX/LPA axis is an important regulator of lymphocyte extravasation at HEVs. Upon inhibition of this axis, lymphocytes rapidly accumulated in the EC and sub-EC layers, consistent with prolonged lymphocyte residence in this area and delayed egress into the LN parenchyma. LPA counteracted this inhibition, apparently directing lymphocyte egress from the HEVs. Thus, our results delineate an additional level at which lysophospholipids act to promote lymphocyte trafficking: sphingosine 1–phosphate regulates lymphocyte egress from the lymphatic sinus (44), and the ATX/LPA axis regulates lymphocyte egress from the HEVs. We are now generating conditional knockout animals that lack ATX expression selectively in the HEVs to further examine the biological importance of the ATX/LPA axis in lymphocyte trafficking.

**FIGURE 8.** Possible mode of action of the ATX/LPA axis in the regulation of lymphocyte trafficking across HEVs. **Upper panel,** Lymphocytes migrating across the HEVs may follow multiple steps: (1) tethering/adhesion to HEV ECs, (2) intra-EC infiltration, (3) retention in the sub-EC space, (4) retention in the perivenular channel, and (5) egress from HEVs. The ATX/LPA axis is dispensable for lymphocyte infiltration into the EC layer (steps 1 and 2) but indispensable for the lymphocyte extravasation from HEVs (steps 3, 4, and 5). ATX secreted by HEV ECs and neighboring cells appears to be immobilized on the HEV EC surface, at least in part via glycosaminoglycan chains, such as heparan sulfate. The EC-captured ATX converts circulating LPC to LPA in situ; LPA, in turn, induces their motility and permeability, as well as lymphocyte detachment from the EC, which may collectively drive directional lymphocyte movement from the basal laminal aspect of HEV ECs to the surrounding LN parenchymal compartment. **Lower panel,** Inhibition of ATX or LPA receptors results in lymphocyte accumulation in the EC and the sub-EC layers, thus inhibiting lymphocyte migration into the LN parenchyma. Collectively, the ATX/LPA axis appears to serve as an endogenous regulator of lymphocyte extravasation at HEVs.
References


**Supplemental Data**

Supplemental data include 4 movies.

**Movie S1: The ATX/LPA axis inhibitor BrP-LPA and 3ccPA reduce the lymphocyte extravasation from HEVs (mov video, 3.6 MB) (Supplemental to Figure 5E)**

Representative two-photon microscopic images obtained from the inguinal LN of vehicle-treated mice. GFP⁺ splenic lymphocytes (green) were i.v. injected into mice that had been injected with BrP-LPA (200 μM), 3-ccPA (500 μM), Ki16425 (200 μM), or PBS into the footpad. Blood vessels were visualized by i.v. injection of rhodamine-dextran (red), and HEVs were identified by their unique morphology. Time is shown in minutes and seconds. Events are accelerated 90x over real time.

**Movie S2: The ATX inhibitor HA130 inhibits lymphocyte release from an HEV EC, and LPA abrogates HA130’s inhibitory effect, allowing lymphocytes to interact with HEV ECs dynamically (mov video, 9.7 MB) (Supplemental to Figure 7B)**

LN stromal cells were plated on an 8-well chambered cover glass coated with collagen I, and incubated for 4 hrs in serum-containing medium. After non-adherent cells were gently removed, the HEV ECs identified by MECA-79 staining were video-recorded under 1,000× magnification. In this movie, two to three lymphocytes were continually
locomoting underneath a single HEV EC. Occasionally, some of them moved out from the EC, whereas some lymphocytes crawled underneath the EC (left). The identical HEV ECs were incubated in the presence of HA130 (500 nM) for 30 min. HA130 inhibited mainly the release of lymphocytes that had migrated underneath HEV ECs (middle). The identical HEV ECs were further incubated with LPA (20 μM). LPA abrogated the HA130’s inhibitory effect, allowing lymphocytes to interact with EC dynamically again (right). Lymphocytes in the act of ingress to and egress from the EC are shown with arrows in green and red, respectively, whereas those unable to translocate the main cell body to the outside of the ECs, and thereby failed to egress from the ECs are shown with blue arrows.

**Movie S3: Blebbistatin treatment severely attenuates lymphocyte release from HEV ECs and inhibits membrane ruffle formation of ECs (mov video, video 9.0 MB). (Supplemental to Figure 7C and 7D)**

Upper panels: LN stromal cells and lymphocytes were incubated in serum-containing medium, and HEV ECs were identified by MECA-79 staining. The interaction of HEV ECs and lymphocytes were recorded for 20 min (left) and further monitored in the presence of a myosin II inhibitor blebbistatin (50 μM, right). Lower panels: HEV ECs were incubated in serum-containing medium and recorded for 20 min (left). The same
HEV ECs were further monitored for surface membrane ruffling in the presence of blebbistatin for 20 min (right). Lymphocytes in the act of ingress to and egress from the EC are shown with arrows in green and red, respectively, whereas those unable to translocate the main cell body to the outside of the ECs, and thereby failed to egress from the ECs are shown with blue arrows.

**Movie S4: Blebbistatin acts on HEV ECs to inhibit lymphocyte release from the HEV ECs (mov video, 4.8 MB). (Supplemental to Figure 7E)**

LN stromal cells and lymphocytes were incubated in serum-containing medium and treated with vehicle (DMSO, left) or blebbistatin (right) for 30 minutes. After unbound cells were gently washed off, untreated GFP⁺ lymphocytes were added, and lymphocyte-HEV EC interactions were monitored. HEV ECs were identified by MECA-79 staining. GFP⁺ lymphocytes in the act of ingress to and egress from the EC are shown with arrows in green and red, respectively, whereas those unable to translocate the main cell body to the outside of the ECs, and thereby failed to egress from the ECs are shown with blue arrows.