Involvement of the Lysophosphatidic Acid-Generating Enzyme Autotaxin in Lymphocyte-Endothelial Cell Interactions

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Autotaxin (ATX) is a secreted protein with lysophosphatidic acid (LPA) from lysophosphatidylcholine. Here we report that functional ATX is selectively expressed in high endothelial venules (HEVs) of both lymph nodes and Peyer’s patches. ATX expression was developmentally regulated and coincided with lymphocyte recruitment to the lymph nodes. In adults, ATX expression was independent of HEV-expressed chemokines such as CCL21 and CXCL13, innate immunity signals including those via TLR4 or MyD88, and of the extent of lymphocyte trafficking across the HEVs. ATX expression was induced in venules at sites of chronic inflammation. Receptors for the ATX enzyme product LPA were constitutively expressed in HEV endothelial cells (ECs). In vitro, LPA induced strong morphological changes in HEV ECs. Forced ATX expression caused cultured ECs to respond to lysophosphatidylcholine, up-regulating lymphocyte binding to the ECs in a LPA receptor-dependent manner under both static and flow conditions. Although in vivo depletion of circulating ATX did not affect lymphocyte trafficking into the lymph nodes, we surmise, based on the above data, that ATX expressed by HEVs acts on HEVs in situ to facilitate lymphocyte binding to ECs and that ATX in the general circulation does not play a major role in this process. Tissue-specific inactivation of ATX will verify this hypothesis in future studies of its mechanism of action. (Am J Pathol 2008, 173:1566–1576; DOI: 10.2353/ajpath.2008.071153)

Much of the efficiency of the immune system depends on continual lymphocyte recirculation through the secondary lymphoid tissues. Lymphocyte recirculation begins with blood lymphocytes interacting with the endothelial wall of high endothelial venules (HEVs) in lymph nodes (LNs) and Peyer’s patches (PPs), in a process called lymphocyte rolling. The rolling brings lymphocytes into contact with HEV endothelial cells (ECs), which express a variety of chemokines and adhesion molecules. Lymphocytes bind to these molecules and transmigrate through the HEVs.1,2 This interaction between lymphocytes and the molecules on HEV ECs is transient and reversible, and both lymphocytes and HEV ECs show marked and coordinated morphological changes during the process,3 indicating that each of the two cell types acts on the other. The molecular details behind these interactions, however, remain to be fully elucidated.

Accumulating evidence indicates that a variety of lysophospholipids act on immune cells by transmitting signals through a family of G-protein-coupled receptors to control the cells’ differentiation, motility, and survival.4 In

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particular, structurally related lysosphospholipid mediators such as sphingosine-1-phosphate (S1P) and lysosphosphatidic acid (LPA), which can be generated from lipid stores by cellular enzymatic pathways in macrophages, dendritic cells, mast cells, and platelets, act on lymphocytes and dendritic cells through members of the S1P-receptor family (S1P1 ~ S1P5) and the LPA-receptor family (LPA1 ~ LPA3), respectively.4–7 Among these ligand-receptor interactions, S1P and its receptor S1P1 are required for thymocyte emigration8 as well as lymphocyte egress from the secondary lymphoid tissues,8,9 although exactly where and how these signaling molecules function is not fully understood. In addition, the functional significance of the interaction between LPA and its receptors in the immune system is unknown.

Autotaxin (ATX), originally identified as a tumor motility-stimulating protein,11 is a secreted protein identical to lysosphospholipase D (lysoPLD).12 In vitro, ATX converts lysosphosphatidylcholine (LPC) and sphingosylphosphorylcholine to LPA12 and S1P13, respectively. However, a recent study indicates that ATX is a major producer of LPA, but not of S1P, in vivo.14 Under physiological conditions, ATX is expressed in the central nervous system and is implicated in oligodendrocyte function.15 In addition, ATX transcripts have been found in human tonsillar HEVs,16 but its protein expression has not been reported in HEVs, let alone its function. While this manuscript was under review, Kanda and colleagues17 published that ATX is involved in lymphocyte trafficking from blood into lymphoid tissues.

Here we report that ATX is preferentially expressed in the HEVs of mouse LNs and PPs and that its expression correlates positively with the ability of venules to support lymphocyte trafficking across the endothelial walls. ATX was clearly inducible in chronically inflamed venules that mediate pathological lymphocyte trafficking. Interestingly, LPA receptors were expressed in HEV ECs, and the addition of LPA caused substantial cytoskeletal changes. Forced ATX expression made cultured ECs reactive to LPC, which up-regulated lymphocyte binding to the ECs in a LPA receptor-dependent manner. Because ATX is a major producer of LPA, these results support the idea that ATX and LPA are active participants in lymphocyte trafficking, presumably regulating lymphocyte migratory behaviors locally at HEVs.

Materials and Methods

Mice

C57BL/6J and AKR/J mice were purchased from Japan SLC (Hamamatsu, Japan) and CLEA Japan (Tokyo, Japan), respectively. NOD and GFP-transgenic mice18 were kind gifts from Dr. J. Miyazaki (Division of Stem Cell Regulation Research, Osaka University, Osaka, Japan) and Dr. M. Okabe (Research Institute of Microbial Diseases, Osaka University), respectively. The following genetically engineered and mutant mice were backcrossed on a C57BL/6J background for at least four generations, and the backcrossed progeny were used in experiments at 6 to 12 weeks of age: CCR7−/−,19 DOCK2−/−,20 TLR2−/−,21 TLR4−/−,21 MyD88−/−,21 CXCL13−/−,22 and plt/plt.23 Mice were housed at the Institute of Experimental Animal Sciences at Osaka University Medical School, and the experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the Osaka University Graduate School of Medicine.

Cells and Reagents

MAdCAM-1+/− HEV ECs were purified from pooled mesenteric LNs as described.24 The mouse capillary EC line, MBEC425, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum (FCS), 1 mmol/L sodium pyruvate, 10 mmol/L HEPEs, 0.1 mmol/L nonessential amino acids, and 50 μmol/L 2-mercaptoethanol. To establish cells stably expressing ATX, MBE4 cells were co-transfected with a mixture of two plasmids, mylsoPLDpCAGGS26 and pSV2-neo, carrying the neomycin (G418) resistance gene, and grown in DMEM containing 750 μg/ml of G418 (Sigma, St. Louis, MO). Individual G418-resistant clones were isolated by limiting dilution and screened by Western blotting and immunocytochemistry with an anti-ATX mAb (4F1)14 and by measuring the lysoPLD activities of the culture media. Fatty acid-free bovine serum albumin (BSA), 1-oleoyl-LPC, and Ki16425 were purchased from Sigma. LPA (1-oleoyl-2-hydroxy-sn-3-glycerol-3-phosphate) was purchased from Biomol International (Plymouth Meeting, PA).

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from various tissues using Isogen (Nippongene, Toyama, Japan) and reverse-transcribed using the Super-Script first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). Oligonucleotide primers for PCR were designed using Primer Express Software (Applied Biosystems, Foster City, CA). The sequences of the oligonucleotides used in PCR were as follows: ATX (mouse), forward, 5’-GGAGAATACAGTGGTAGATGATG-3’; ATX (mouse), reverse, 5’-ACGGAGGGCCGGACAAAC-3’, GAPDH, forward, 5’-GGCAAGGTGCATCCATGCAACT-3’, GAPDH, reverse, 5’-GAGGGCCATCCACAGTCTT. PCR reactions were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems). The transcript number of GAPDH was quantified, and each sample was normalized on the basis of GAPDH content.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (4 μm) were dewaxed, rehydrated, and boiled in 10 mmol/L citrate buffer, pH 6.0. Sections were then washed in Tris-buffered saline and incubated with anti-ATX mAb (4F1) and anti-PNAd mAb (MECA-79)27 or anti-MAdCAM-1 mAb.
(MECA-89)28 at 4°C overnight, followed by biotin-conjugated goat anti-rat IgG + IgM (Southern Biotechnology Associates, Inc., Birmingham, AL). The sections were further incubated with horseradish peroxidase-conjugated ABC reagent (Vectorstain ABC-HRP kit; Vector Laboratories, Burlingame, CA), developed with Metal Enhanced DAB (Pierce, Rockford, IL), and counterstained with Mayer’s hematoxylin (Muto Pure Chemicals Co., Tokyo, Japan). In some experiments, biotin-labeled tyramide reagent (PerkinElmer Life Sciences, Boston, MA) was used for signal amplification.

**PCR Analysis**

Total RNA was extracted from mesenteric LNs using TRIzol (Invitrogen). Single-strand cDNA synthesized using the Ready-To-Go kit (GE Health Care UK Ltd., Amersham Place, UK) and a cDNA library of MAdCAM-1+ HEVs29 were used in PCR analysis with Ex-TaqDNA polymerase (Takara Bio, Otsu, Japan). PCR was performed at 94°C for 2 minutes, 38 cycles at 94°C for 30 seconds, at 57°C for 30 seconds, at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The following primer pairs were used: ATX: sense, 5’TCTAGCAGTTCCAGACACCTG-3’, antisense, 5’GGTGGGGATGGATCCATG-3’; LPA1: sense, 5’-AAGAAAGCACATGTGTTGTG-3’, antisense, 5’- ATGCTATAGGACATCGTG-3’; LPA2: sense, 5’-GTAGTACTGAAAGCTATTCC-3’, antisense, 5’-AGCCTAGCAGTATAGGCCAG-3’; LPA3: sense, 5’-GATGAGAGTCCACAGCAACTTG-3’; LPA4: sense, 5’-ATGGATGACGATATCGCT-3’. PCR products were analyzed by agarose gel electrophoresis.

**Fluorescence Microscopy**

Purified MAdCAM-1+ HEV ECs were seeded in collagen type 1-coated eight-well culture slides (Becton Dickinson, Mountain View, CA) (4 × 10⁴ cells/well) and incubated for 3 hours in DMEM containing 20% FCS (HyClone, Logan, UT). After the nonadherent cells were removed, the remaining ECs were treated for 60 minutes with LPA (15 μmol/L) in fresh DMEM containing FCS stripped with 10% charcoal. Similarly, MBEC4 cells and ATX-expressing MBE4 cells were seeded in Lab-Tek II chamber slides (Nalge Nunc, Rochester, NY) (0.5 × 10⁴ cells/well), cultured overnight, and treated for 60 minutes with LPA or LPC (10 μmol/L) in fresh DMEM containing 0.1% fatty acid-free BSA. In some experiments, cells were pretreated with 10 μmol/L Ki16425 (Sigma) for 60 minutes at 37°C. After two washes in PBS, the cells were fixed in 4% paraformaldehyde, permeabilized in PBS containing 0.1% Triton X-100, and incubated with Alexa Fluor 594-conjugated MECA89 (2 μg/ml) followed by Alexa Fluor 488-phalloidin (1 U/ml) (Invitrogen) (for MAdCAM-1+ HEV ECs) or Alexa Fluor 488-phalloidin alone (for MBEC4 and ATX-expressing MBE4 cells). The cells were then observed under a confocal microscope (LSM510META; Carl Zeiss, Jena, Germany; or Radiance 2100; Bio-Rad, Hercules, CA) using Fluormount-G mounting medium (Southern Biotechnology).

**In Situ Hybridization Assay**

Plasmids (pCRII; Invitrogen) containing cDNA fragments of LPA1 (500 bp), LPA4 (366 bp), or MAdCAM-1 (482 bp) were used as templates for RNA probe synthesis. Digoxigenin (DIG)-labeled antisense or sense probes were prepared with T7 and SP6 RNA polymerase (Applied Biosystems), respectively, using the DIG RNA labeling mix (Roche Diagnostics, Basel, Switzerland). Mesenteric and peripheral LNs were fixed with 4% paraformaldehyde for 2 hours, incubated in 30% sucrose in phosphate-buffered saline (PBS) overnight, and embedded in OCT compound. Eight-μm-thick serial frozen sections were fixed in 4% paraformaldehyde for 20 minutes, incubated in 0.1% H₂O₂, and permeabilized with 50 μg/ml proteinase K for 5 minutes. After an additional fixation with paraformaldehyde, the sections were treated with acetylated saline in triethanolamine for 10 minutes. The sections were then prehybridized with 50% formamide, 5× standard saline citrate, 1 μg/ml yeast tRNA (Roche Diagnostics), 100 μg/ml heparin, 1× Denhardt’s solution, and 0.1% Tween 20 at 60°C for 3 hours, and then hybridized with labeled probe in the same solution overnight at 60°C. After being washed, the sections were incubated with horseradish peroxidase-conjugated anti-DIG (Roche Diagnostics), followed by biotin-labeled tyramide (TSA Biotin System, PerkinElmer Life Sciences) for signal amplification. The hybridized probes were then detected by ABC-alkaline phosphatase (Vector Laboratories) and NBT/BCIP (Roche Diagnostics).

**Lymphocyte Binding Assay**

Freshly isolated MAdCAM-1+ HEV ECs, MBEC4, and ATX-expressing MBE4 cells were cultured and stimulated as follows. The MAdCAM-1+ HEV ECs were plated (2.5 × 10⁴ cells/well) in collagen-coated 96-well plates (Becton Dickinson) and allowed to adhere for 3 hours in DMEM containing 20% FCS (HyClone). After the unbound cells were removed, the remaining cells were starved for 60 minutes by incubation in DMEM containing 0.1% fatty acid-free BSA. In some experiments, ECs were pretreated with 10 μmol/L Ki16425 for 60 minutes at 37°C. After two washes in PBS, the cells were fixed in 4% paraformaldehyde, permeabilized in PBS containing 0.1% Triton X-100, and incubated with Alexa Fluor 594-conjugated MECA89 (2 μg/ml) followed by Alexa Fluor 488-phalloidin (1 U/ml) (Invitrogen) (for MAdCAM-1+ HEV ECs) or Alexa Fluor 488-phalloidin alone (for MBEC4 and ATX-expressing MBE4 cells). The cells were then observed under a confocal microscope (LSM510META; Carl Zeiss, Jena, Germany; or Radiance 2100; Bio-Rad, Hercules, CA) using Fluormount-G mounting medium (Southern Biotechnology).
did not adhere to plastic were labeled with the fluorescent indicator BCECF-AM as described, in DMEM with 0.1% fatty acid-free BSA. They were then added to the wells containing the ECs (5 × 10⁵ cells/well) and allowed to bind to the ECs for 30 minutes under static conditions or with rotation (120 rpm). Unbound lymphocytes were removed by inverting the plate, which had been filled with prewarmed FCS-free RPMI 1640 and sealed with Parafilm (Pechinery Plastic Packaging, Inc., Menasha, WI) for 30 minutes at room temperature. The adherent lymphocytes were solubilized with 1% Nonidet P-40 in PBS, and fluorescence intensity was measured by a fluorescence enzyme-linked immunosorbent assay reader (SpectraMax Gemini XS; Molecular Devices, Sunnyvale, CA).

**Short-Term Homing Assay**

The lymphocyte migration assay was performed as previously described. Briefly, spleen cells obtained from GFP-transgenic mice were injected intravenously into mice (2 × 10⁷ cell/mouse) that had received an intraperitoneal injection of a cocktail of anti-ATX mAbs (clones 5E5, S13A9, and S9A9; 150 μg each) or rat IgG (450 μg/mouse; Cappel, Aurora, OH) 3 hours before. The mice were sacrificed 2.5 hours after the lymphocyte injection, and the spleen, mesenteric LNs, brachial LNs, and PPs were harvested. The number of lymphocytes that had migrated into these tissues was determined by flow cytometry.

**LysoPLD Assay**

Plasma samples (10 μl) were incubated with 4 mmol/L LPC in buffer containing 100 mmol/L Tris-HCl (pH 9.0), 500 mmol/L NaCl, 5 mmol/L MgCl₂, and 0.05% Triton X-100 for 12 hours at 37°C. The liberated choline was detected by an enzymatic photometric method using choline oxidase (Asahi Chemical Industry, Tokyo, Japan), horseradish peroxidase (Toyobo, Tokyo, Japan), and TOOS reagent [N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine] (Dojin, Tokyo, Japan), as reported previously. Absorbance was read at 560 nm and converted to nanomoles of choline by comparison to a choline standard curve.

**Statistical Analysis**

A Student’s t-test was applied to compare the statistical difference within two groups.

**Results**

**ATX Is Constitutively and Selectively Expressed in HEVs of Peripheral and Mesenteric LNs and PPs**

Preliminary analysis showed that ATX transcripts appeared at moderate frequencies in the previously generated 3’-directed cDNA libraries of PNAd⁺ HEV ECs and of MadCAM-1⁺ HEV ECs, they appeared five times among 1558 transcripts obtained from the PNAd⁺ HEV cDNA library and five times among 2101 transcripts in the MadCAM-1⁺ HEV cDNA library. Transcripts of endoglin were found at comparable frequencies in these libraries (data not shown). ATX transcripts are also found in human tonsil cDNA library enriched for HEV-derived cDNAs by subtraction with lymphocyte cDNAs and umbilical vein endothelial cDNAs. Real-time PCR analysis showed that ATX mRNA was strongly expressed in the peripheral and mesenteric LNs as well as in the brain and kidney among the mouse tissues tested (Figure 1A). These findings prompted us to look for the expression and localization of ATX proteins in HEVs. As shown in Figure 1B, specific mAb against ATX readily detected its expression in HEVs of peripheral and mesenteric LNs and PPs, but not in non-HEV blood vessels in experimentally naïve mice. Although staining intensities varied in different HEVs, a majority of HEVs of LNs appeared positive for ATX expression, whereas ~30 to 40% of HEVs of PPs expressed ATX. In mesenteric LNs, both PNAd⁺ HEVs and MadCAM-1⁺ HEVs expressed ATX (Figure 1C).

We next examined ATX expression in developing LNs. Although ATX expression was not observed prenatally (Figure 2A), it was detectable 1 day after birth (Figure 2B), and its level increased progressively until postnatal day 7 (Figure 2, C and D), concomitant with the increasing numbers of lymphocytes around HEVs in the peripheral and mesenteric LNs. The appearance of ATX also paralleled to that of PNAd in peripheral LNs (data not shown). ATX Expression Is Up-Regulated in Venules at Sites of Chronic Inflammation

HEV-like venules appear in sites other than peripheral and mesenteric LNs and PPs under certain pathological conditions. For instance, a marked increase in lymphocyte trafficking is associated with the appearance of HEV-specific adhesion molecules and a HEV-like morphology in the cortico-medullary venules of the thymus in aged AKR/J mice that develop thymic hyperplasia and lymphoma. We found that a majority of the cortico-medullary venules of hyperplastic thymus from old AKR/J mice were PNAd⁺, as revealed by mAb MECA-79 staining, and ~60 to 70% of the PNAd⁺ venules were also positive for ATX expression (Figure 3A). Most of the ATX⁺ venules appeared PNAd⁺ (Figure 3A). In contrast, a majority of the thymic venules of young AKR/J mice (6 to 8 weeks old) and 30-week-old C57BL/6 mice were PNAd-negative (date not shown), although a small fraction of C57BL/6 mice (~20 ~ 30%) showed weak but distinct ATX expression in these venules at 6 to 8 weeks of age (Figure 3B). A previous study showed that insulitis in NOD mice is associated with the occurrence of HEV-like vessels bearing PNAd epitopes. Our study confirmed this observation and showed that a substantial proportion of the PNAd⁺ venules in the pancreas of the NOD mice were also positive for ATX, whereas the venules of uninflamed islet tissues in the NOD mice were...
negative for PNAd and ATX (Figure 3C). These observations are in agreement with the hypothesis that ATX plays a role in lymphocyte trafficking into chronically inflamed tissues.

**The Expression of ATX Is Independent of Signals Mediated by Lymphoid Chemokines (CCL21 and CXCL13), DOCK2, or MyD88**

We next addressed the regulatory mechanism of ATX expression. To this end, we first investigated ATX expression in the HEVs of CCR7-deficient mice,\(^{19}\) plt/plt mice,\(^{23}\) and CXCL13-deficient mice,\(^{22}\) all of which show substantially reduced lymphocyte homing. In addition, we examined mice deficient in DOCK-2, which plays an essential role in CCR7- and CXCR5-mediated signaling;\(^{20}\) DOCK-2-deficient mice also show severely impaired lymphocyte
trafficking to LNs. All of the mice examined were 6 to 12 weeks old. We saw no differences in ATX expression in the HEVs of the CCR7-deficient, CXCL13-deficient, plt/plt, or DOCK-2-deficient mice, compared with wild-type mice, indicating that lymphocyte trafficking across HEVs per se does not affect ATX expression in these venules (Figure 4A). These results also indicate that ATX expression is independent of lymphoid chemokines, such as CCL21 and CXCL13, and of CCR7-dependent signals in the HEVs of adult animals.

Given that HEVs express TLR4 (M. Kiyomi and T. Tanaka, unpublished observation), which induces a spe-

Figure 4. The expression of ATX is independent of lymphoid chemokine (CCL19/21 and CXCL13), DOCK2, or MyD88-mediated signals. Anti-ATX staining of paraffin sections of mesenteric LNs from CCR7−/−, CXCL13−/−, DOCK2−/−, or plt/plt mice (A) and TLR4−/− or MyD88−/− mice (B). Scale bars = 100 μm.

Figure 3. ATX expression is up-regulated in venules of AKR hyperplastic thymus and in chronically inflamed blood vessels in the NOD pancreas. A and B: Serial paraffin sections of hyperplastic thymus from aged AKR/J mice (A) and thymus of normal mice (B) incubated with anti-PNAd mAb (A: top) or anti-ATX mAb (A: bottom). In A, the right panel shows a higher magnification view of the boxed area at left. In B, right panel, boxed region shows higher magnification view of ATX expression near the cortico-medullary junction in wild-type mouse thymus (indicated by arrow at low magnification). C: Serial paraffin sections of inflamed (top) and noninflamed (bottom) pancreas from NOD mice incubated with anti-PNAd mAb or anti-ATX mAb, as indicated. Scale bars = 100 μm.
specific differentiation program in certain cell types on its ligation,38 we next examined whether ATX expression is under the control of TLR signaling. In mice deficient for TLR4 or MyD88, which mediates signaling by many of the TLRs,21 there was no noticeable difference from wild-type in ATX expression (Figure 4B), which is incompatible with the idea that TLR signaling regulates ATX expression in HEVs. These results also indicate that innate immunity signals mediated by TLR4 and MyD88 are dispensable for ATX expression in HEVs.

HEV ECs Express Specific Receptors for LPA and Show Cytoskeletal Changes by LPA Stimulation

Although ATX can generate LPA12,39 and S1P13 from their respective precursors in vitro, recent studies indicate that ATX is the major LPA-producing enzyme in vivo.14,40 Therefore, to investigate whether LPA could act through a paracrine or autocrine mechanism at the HEV EC surface, we next examined LPA receptor expression in HEV ECs. We found that MAdCAM-1-HEV ECs obtained from mesenteric LNs expressed LPA1 and LPA4, and that unfractoned mesenteric LN cells consisting mainly of lymphocytes expressed readily detectable levels of LPA1, LPA2, LPA3, and a low level of LPA4 at the mRNA level (Figure 5A). Importantly, the possibility that contaminating lymphocytes were responsible for the expression of the LPA receptors in the HEV EC preparation was ruled out because the EC preparation used here showed no signal for CD3ε or L-selectin mRNA, whereas the mesenteric LN cells showed strong CD3ε and L-selectin mRNA signals. The LPA receptor expression by HEV ECs was also verified by in situ hybridization analysis. As shown in Figure 5B, MAdCAM-1-expressing HEV ECs showed strong signals when hybridized with antisense probes for LPA1 and LPA4, but not with sense probes for these receptors. These findings collectively indicate that HEV ECs express LPA1 and LPA4.

We next examined the function of LPA receptors expressed by HEV ECs. Given that ATX is constitutively expressed in HEV ECs, one possible role for this cell-motility protein is to induce the local production of LPA in HEVs, which could function in turn as an autocrine or paracrine factor for the HEV ECs and/or lymphocytes. To test this possibility, we added LPA to HEV ECs in vitro and found by confocal imaging that the HEV ECs showed prominent morphological changes in response to the exogenous LPA (Figure 5C); ~50% of HEV ECs showed extended cell morphology with actin-rich cell protrusions and cell-surface ruffling, and this change was almost completely inhibited by Ki16425, which antagonizes at least LPA1 and LPA3, if not other isoforms.51

We also examined the effect of LPA and LPC on lymphocyte binding to HEV ECs. However, because purified HEV ECs showed high endogenous lymphocyte binding in the absence of any deliberate stimulation in vitro24,33 we could not determine the effect of LPA or LPC on lymphocyte binding in this experimental setup. Therefore, to further examine this issue, we resorted to using ATX-expressing cultured ECs that bind lymphocytes only moderately.

Forced Expression of ATX by Cultured ECs Confers Responsiveness to LPC

To generate cultured EC lines constitutively expressing both ATX and LPA receptors, we introduced an expression vector for ATX into an EC line, MBEC4, that endog-
enously expresses receptors for LPA, and determined the cells’ responses to LPA and LPC. As shown in Figure 6A, the ATX-transfected MBEC4 cell lines (MBEC4-ATX) expressed ATX abundantly, whereas the parental MBEC4 ECs did not express ATX. The MBEC4-ATX cells showed strong morphological changes in response to LPA and LPC (70% of cells showed extended cell morphology with well-developed stress fibers), and these

Figure 6. LPC induced morphological changes in ATX-expressing ECs and enhanced lymphocyte binding to them. A: Expression of ATX in MBEC4-ATX cells analyzed by Western blotting. Essentially all MBEC4-ATX cells (>98%) were positive for ATX expression and they produced biologically active ATX with lysoPLD activity (241.8 nmol/ml/hour in 100-hour conditioned medium). B: MBEC4 or MBEC4-ATX cells incubated with or without 10 μmol/L LPA or LPC were fixed, permeabilized, and stained with Alexa Fluor 488-phalloidin. For inhibition studies, cells were pretreated with 10 μmol/L Ki16425, an LPA1 and LPA3 antagonist. C and D: MBEC4-ATX and MBEC4 incubated with or without various concentrations of LPA or LPC to which BCECF-labeled lymphocytes were added without (C) or with continuous rotation (120 rpm) (D). The adherent lymphocytes were solubilized and quantified by a fluorescence plate reader. For inhibition studies, these cells were pretreated with Ki16425. Data represent the mean ± SD for three separate experiments. * P < 0.005 compared with vehicle-treated MBEC4-ATX cells. ** P < 0.005 compared with vehicle-treated MBEC4 cells.
changes were inhibited by Ki16425 (Figure 6B). In contrast, the parental MBEC4 cell line responded to LPA but not to LPC. These results are in agreement with the hypothesis that ECs producing high levels of ATX respond not only to LPA but also to LPC by converting LPC to LPA, whereas ATX-negative cells respond to LPA but not to LPC, because they are incapable of hydrolyzing LPC to LPA.

Differential effects by LPA and LPC on lymphocyte binding to these EC lines were also observed (Figure 6C). The MBEC4-ATX cells showed increased lymphocyte binding when exposed to 1 to 10 μmol/L LPA or LPC under static conditions. In contrast, the MBEC4 cells showed increased lymphocyte binding in response to 1 to 10 μmol/L LPA but not LPC. Under continuous rotation (120 rpm), the MBEC4-ATX showed increased lymphocyte binding in the presence of LPC, whereas the parental MBEC4 cells did not (Figure 6D). These results further support the hypothesis that endothelial ATX promotes the motility of lymphocytes and ECs by converting LPC to LPA on the EC surface.

Depletion of ATX from the Circulation Does Not Impair Lymphocyte Trafficking to Secondary Lymphoid Tissues

ATX is a type-II transmembrane protein that undergoes membrane-proximal cleavage to yield a soluble lysoplD that is released into the plasma.42 To address the functional roles of ATX in regulating lymphocyte trafficking in vivo, we attempted to deplete the ATX by injecting a cocktail of anti-ATX mAbs intraperitoneally into mice. This injection successfully depleted ATX from the plasma, decreasing the plasma lysoplD activity to ~10% of that seen in mice given a control rat IgG (Figure 7A). However, lymphocyte trafficking to the spleen, brachial LNs, mesenteric LNs, and PPs in the anti-ATX-treated mice, as examined by adoptive transfer of fluorescently labeled lymphocytes, was comparable to the trafficking observed in the control mice (Figure 7B). These observations suggest that the ATX in the general circulation does not play a major role in lymphocyte trafficking to secondary lymphoid tissues but argues for the role of ATX in the local environment; given that ATX is selectively expressed in HEVs and that endothelial ATX promotes the motility of lymphocytes and ECs as shown above, these findings are compatible with the idea that ATX regulates lymphocyte-endothelial interactions not systemically but locally at HEV.

Discussion

In this study, we addressed the biological role of ATX expressed by HEVs. ATX expression was abundant in adult HEVs but scarce in the HEVs of newborns. In the developing LNs, ATX expression rapidly increased postnatally in parallel with PNAd expression, and was concomitant with the appearance of numerous lymphocytes around the HEVs. Although not expressed in intact venules of nonlymphoid tissues, ATX appeared in certain venules of chronically inflamed tissues, such as the pancreatic venules of NOD mice and the thymic venules of AKR mice, which expressed the L-selectin-reactive PNAd epitope and showed prominent perivascular lymphocyte infiltration. In contrast, ATX was absent from blood vessels in these tissues that showed no perivascular lymphocyte infiltration. Thus, ATX expression was always positively correlated with lymphocyte trafficking across venules, strongly implicating ATX in this phenomenon.

Kanda and colleagues17 recently published a study in which they showed that ATX, secreted by HEVs, binds to the chemokine-activated lymphocytes and generated LPA, which in turn enhances lymphocyte motility through Gai-coupled receptors. They also showed that intravenous injection of an enzymatically inactive form of ATX blocked the trafficking of blood lymphocytes to LNs, PPs, and spleen.17 Our in vitro observations also support a functional role for ATX in lymphocyte-HEV interactions.
Because ATX is a major producer of LPA, we hypothesized that ATX generates LPA from its precursor LPC and that LPA may act on not only lymphocytes but also HEV ECs in an autocrine and/or paracrine manner, because both cells express LPA receptors. Consistent with this idea, HEV ECs expressed LPA, and LPA, and although the effect of LPA and LPC on lymphocyte binding to HEV ECs could not be determined because of a high background lymphocyte binding, the HEV ECs showed increased cell-surface ruffling and pseudopod extension in response to exogenous LPA, which was abrogated by Ki16425, indicating that LPA enhances the motility of HEV ECs, which may facilitate physical encounters and subsequent interactions with lymphocytes in situ. In addition, when ATX-expressing cultured ECs were used, it was notable that pretreatment with not only LPA but also LPC increased the lymphocyte binding to ATX-expressing ECs under static as well as flow conditions. In contrast, only LPA (not LPC) increased lymphocyte binding in ATX-negative ECs. These observations support the idea that ECs producing high levels of ATX respond to LPA and also to LPC and that endothelial ATX enhances the motility of lymphocytes and ECs by converting LPC to LPA on the HEV EC surface locally. Whether ATX has an effect on lymphocyte binding to HEVs could be finally resolved by in situ application of ATX inhibitors, such as neutralizing anti-ATX monoclonal antibodies, which are as yet unavailable, to HEVs.

Given that ATX is expressed in restricted cell types in vivo, its expression must be regulated rigorously. Although transcriptional regulation is likely to be one mechanism for this, we do not know what molecules are involved in regulating the transcription of ATX. Our experiments with mice genetically deficient in CXCL13, DOCK-2, CCR7, TLR4, or MyD88, and with plt/plt mice, indicated that neither signals from lymphoid chemokines, such as CCL21 and CXCL13, nor the TLR4 are involved in the regulation of ATX expression. Lymphocyte trafficking itself did not appear to be involved in this process either.

Whereas the above-mentioned results are compatible with the idea that ATX is involved in the up-regulation of lymphocyte-HEV interactions by generating LPA locally, we found that the systemic depletion of ATX did not affect lymphocyte trafficking into the LNs. This shows a contrast to the study by Kanda and colleagues in which they showed that enzymatically inactive ATX injected intravenously had an inhibitory effect on short-term lymphocyte trafficking to lymphoid tissues. This discrepancy might be at least in part attributable to the fact that the mutant ATX is much smaller in size and probably penetrates into tissue parenchyma more readily than antibodies. Given that ATX is produced by limited cell types in vivo, we envisage that ATX acts locally at the surface of these cells, and the circulating level of ATX may be irrelevant to ATX’s function in vivo. However, because ATX knockout mice are embryonically lethal, we are currently unable to further assess the in vivo function of ATX at HEVs. Conditional expression or deletion of ATX in HEV ECs will be required to answer this issue.

Recent studies have shown that ATX also plays a critical role for blood vessel formation during development. ATX-deficient embryos show severe vascular defects and die at midgestation. In the fetal stage, ATX mRNA is mainly detected in endodermal cells surrounding yolk sac and secreted ATX protein accumulates in amniotic fluid. Although ATX and LPA themselves are not angiogenic in vitro, they can strongly promote blood vessel formation by stabilizing preformed blood vessels through currently unknown mechanisms. In the adult stage, we showed that ATX is selectively expressed in HEV ECs as well as ECs of certain blood vessels in chronically inflamed tissues. This raises the possibility that ATX, in addition to stimulating cellular interactions between lymphocytes and ECs, contributes to the formation of specialized blood vessels, which support an increased lymphocyte trafficking in LNs and PPs as well as chronically inflamed tissues by regulating production of LPA in situ. Mechanisms underlying the selective expression of ATX in the ECs of certain blood vessels and LPA receptors responsible for vascular formation remain unknown. Further study is clearly required to resolve this issue.

Collectively, these results support the hypothesis that ATX enhances lymphocyte-HEV interactions in vivo. ATX may provide another layer of regulation, in addition to that by lymphoid chemokines, to the trafficking of lymphocytes across HEVs. Although it remains to be verified whether ATX mediates lymphocyte-HEV interactions via LPA, the future use of specific inhibitors of these molecules, which are not yet available, should finally elucidate the mode of action of ATX at the HEV-lymphocyte interface.

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