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Autotaxin, a lysophosphatidic acid-producing ectoenzyme, promotes lymphocyte entry into secondary lymphoid organs

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Abstract

The extracellular lysophospholipase D, autotaxin (ATX), and its product lysophosphatidic acid (LPA) have diverse roles in development and cancer, but little is known about functions in the immune system. We found that ATX was highly expressed in high endothelial venules (HEVs) of lymphoid organs and was secreted. Chemokine-activated lymphocytes expressed enhanced receptors for ATX, providing a mechanism to target the secreted ATX onto lymphocytes undergoing recruitment. LPA induced chemokinesis in T-cells. Intravenous injection of enzymatically inactive ATX attenuated homing of T-cells to lymphoid tissues, likely by competing with endogenous ATX and exerting a dominant-negative effect. Our results support a novel and general step in the homing cascade, in which the ectoenzyme ATX facilitates lymphocyte entry into lymphoid organs.

Introduction

High endothelial venules (HEVs) in lymph nodes, Peyer's patches and other secondary lymphoid organs contribute to continuous immune surveillance by supporting lymphocyte recruitment from the blood^{1–3}. Considerable work has established that HEVs are morphologically and functionally specialized to capture lymphocytes circulating at high speed in the bloodstream and to support their migration into the lymphoid organ. HEVs are characterized by endothelial cells (HECs) with a plump morphology together with a well-developed Golgi complex and rough endoplasmic reticulum¹. These features suggest highly active biosynthetic activities, while endothelial cells in other tissues generally have thin and quiescent phenotypes. Sites of chronic inflammation can develop HEV-like vessels that also serve as a gateway for lymphocyte entry^{2,4}.

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Competing interests statement

The authors declare that they have no competing financial interests.

As is generally true for leukocyte-endothelial interactions, lymphocyte-HEV interactions occur in three sequential steps: rolling of lymphocytes along the endothelium, arrest on the endothelium and transmigration across the endothelium^{1,3,5}. In lymph node HEVs, the rolling step, is governed by weak and transient interactions between L-selectin on the lymphocyte and the carbohydrate determinant 'sialyl 6-sulfo Lewis X', which is presented by a family of sialomucin proteins on HEVs². In homing to Peyer's patches, both L-selectin and the integrin $\alpha_4\beta_7$ interact with MAdCAM-1 on HEVs to support lymphocyte rolling⁶. The arrest step is induced by so-called 'arrest' chemokines, such as CCL21 and CCL19 (also known as SLC and ELC, respectively), which are immobilized and presented on the apical aspect of HEVs^{3,7}. These molecules trigger the activation of the integrin LFA-1 on lymphocytes and increase its affinity for its ligands ICAM-1 and ICAM-2 expressed on HEVs. The transendothelial migration step is poorly understood.

The gaps in our knowledge about HEV function have prompted several gene profiling analyses of purified HECs from lymphoid organs^{8–10}. Our previous EST (expressed sequence tag) study of human tonsillar HEC gene expression showed that ~5% of the transcripts encoded ATX¹⁰. ATX was originally identified in human melanoma cell culture medium as an autocrine motility factor, which potently induces motility of the melanoma cells^{11,12}. ATX is overexpressed in various tumors and has been implicated in tumor cell migration, invasiveness and metastasis¹³. ATX is required for normal development, since gene-targeted mice die *in utero* with profound defects in vascular formation and other abnormalities^{14,15}. Notable in the sequence of ATX is a phosphodiesterase domain, which is required for its motility stimulating activity^{12,16}. Several years following its cloning, ATX was found to have lysophospholipase D (lysoPLD) activity, which generates lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC)^{17,18}. As an extracellular lysophospholipid, LPA elicits a wide variety of responses in many cell types through interactions with a family of five G-protein coupled receptors (GPCRs)^{13,19}. Among its most extensively studied activities, LPA regulates cytoskeletal organization and migration in multiple cell types¹³. In several cases, it has been established that the motility-stimulating activity of ATX is mediated through the production of LPA and signal transduction through its specific GPCRs^{17,20}.

Most of the previous interest in ATX has been directed at its functions in cancer and early development. The discovery that transcripts for ATX are highly expressed in HECs¹⁰ prompted our investigation of the potential functions of the protein in the immune system. Our studies reveal a general contribution of ATX to the entry of lymphocytes from the blood into secondary lymphoid organs.

Results

ATX expression in mouse lymph nodes

To explore the expression of ATX transcripts in lymphoid organs, we carried out real-time PCR analysis on total RNA isolated from a range of mouse tissues. Consistent with previous reports, we found that central nervous tissues, such as brain and spinal cord, expressed ATX transcripts more abundantly as compared to most other tissues (Fig. 1a)¹³. However, mesenteric and peripheral lymph nodes (MLNs and PLNs) showed the highest expression of all of the organs tested. Notably, purified HECs from lymph nodes had 11-fold higher expression than whole MLNs. The mouse endothelial cell line, bEnd.3, also exhibited the transcripts but much less than in HECs. There was no detectable expression in SVEC, another mouse endothelial cell line.

We next carried out *in situ* hybridization to localize the ATX transcripts in mouse lymph nodes. Consistent with EST results obtained with human HECs¹⁰ and the real-time PCR measurements (Fig. 1a), an *Autotaxin* (*Enpp2*) antisense probe strongly hybridized to HEVs

of peripheral lymph nodes (Fig. 1b). The probe also selectively reacted with HEVs of Peyer's patches, a gut-associated lymphoid organ (not shown). In spleen, a lymphoid organ which lacks HEVs, hybridization signals were strongest in marginal zone regions, a major site for lymphocyte entry into white pulp areas²¹ (Fig. 1b).

We also carried out an immunohistochemical analysis to determine ATX protein localization in these tissues. We employed an affinity-purified rabbit antibody directed against a 20 amino acid peptide within ATX (Supplementary Fig. 1 online). To assist in the identification of HEVs in lymph node, we took advantage of MECA-79, a mAb that stains HEVs through its recognition of L-selectin ligands². ATX was strongly expressed in MECA-79⁺ HEVs in both MLNs and PLNs (Fig. 2). Peyer's patch HEVs were also positive for ATX (data not shown). Some smaller vessel-like structures, which were negative for MECA-79 and lacked a high wall morphology, were positive for ATX (Fig. 2, arrows). These structures were confirmed as vessels by positive staining for the endothelial marker CD31 (data not shown). In the spleen, central arterioles (CD31⁺) and marginal zones were strongly positive for ATX. To investigate ATX expression at sites of lymphoid neogenesis (so-called tertiary lymphoid organs), we employed NOD and RIP-BLC mice models in which pancreata exhibit HEV-like vessels in association with lymphoid accumulations⁴. We detected ATX expression in MECA-79⁺ HEV-like vessels in both models (Supplementary Fig. 2 online). In all cases, staining was not present when the ATX antibody was incubated with the peptide immunogen or when an isotype control antibody was employed (Supplementary Fig. 1 online).

Secretion of ATX by HECs

Until recently, ATX was thought to be a type II membrane protein, which could be released extracellularly by proteolysis of a plasma membrane-associated precursor¹³. However, ATX has now been shown to be a true secretory protein with a cleavable signal sequence^{22,23}. To determine whether HECs secrete ATX, we isolated this population of cells from disaggregated mouse lymph nodes using immunomagnetic beads coupled to MECA-79. Conditioned medium from cultured HECs was subjected to SDS-PAGE and analyzed by immunoblotting with the ATX antibody (Fig. 3a). The HEC culture supernatant and the crude stromal preparation exhibited a reactive protein of 110 kDa, which is the appropriate mass predicted for ATX. This signal was not present in medium conditioned by the HEC-depleted stroma or by lymphocytes. GlyCAM-1, a secretory product of HECs², was used as a positive control. To establish that the reactive component was indeed ATX, we isolated a cDNA encoding ATX from an HEC cDNA library and transfected it into COS-7 cells. Immunoblotting of the purified recombinant ATX showed a prominent band, which migrated almost identically with the reactive species from HEC culture supernatant. These observations show that HECs synthesize and secrete ATX and establish that these cells are a major ATX-producing population within lymph nodes.

We next addressed whether the secretion of ATX from HECs occurred preferentially from the apical or basolateral surface of the cells. As purified HECs do not organize into a continuous monolayer and rapidly lose their specialized gene expression pattern in culture²⁴, we employed MDCK cells. These cells form a highly polarized and tight monolayer and are widely used to study polarized secretion in epithelial cells²⁵. We prepared a stable MDCK transfectant, which expressed N-terminally His-tagged ATX. These cells established a tight monolayer, as confirmed by measuring the passage of fluorescein isothiocyanate (FITC)-dextran across the cell layer (Fig. 3b). We collected culture medium from above (apical) and below (basolateral) the monolayer and measured ATX by both immunoblotting and enzymatic activity (lysoPLD). By both measures, ATX was predominantly (>90%) secreted into the apical compartment (Fig. 3c,d). We examined an endogenous protein, clusterin (also known as ApoJ), which is secreted in the apical direction by MDCK cells²⁵. We found that 70–80% of clusterin was in the top compartments for both parental cells and the ATX-transfected line. From these results, we

conclude that HECs secrete ATX apically and thus would add the protein to the blood. In fact, ATX is known to be present in blood plasma and serum and these fluids have served as sources for its purification^{17,18}. It remains to be determined whether HEVs in the various lymphoid organs, as well as the positive vessels in the spleen, comprise the major sources of ATX in the blood.

Binding of ATX to lymphocytes

Since our results argue for the apical secretion of ATX from HEVs, we next asked whether a mechanism exists to target the activity of this ectoenzyme onto lymphocytes undergoing recruitment into lymphoid organs. ATX possesses a number of potential integrin-binding motifs including an RGD sequence for interaction with $\alpha_5\beta_1$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$ and an LDV sequence for binding of $\alpha_4\beta_1$ 26. We first explored whether soluble ATX could bind to peripheral blood lymphocytes in suspension. Using a flow cytometry-based assay, we were able to detect binding to these cells in the presence of Mn^{2+} (a general enhancer of integrin activity²⁶) in some experiments but the signal was highly variable (not shown). We next determined if ATX could serve as binding substrate for lymphocytes in a standard adhesion assay for integrin-dependent interactions in which ATX was coated onto plastic wells. Jurkat T cells showed pronounced adhesion to immobilized ATX in the presence of Mn^{2+} and much less interaction when Mn^{2+} was omitted (Fig. 4a). Consistent with previous findings, Mn^{2+} also enhanced Jurkat cell binding to ICAM-1 and VCAM-1. Antibodies against the α_4 and β_1 subunits of $\alpha_4\beta_1$ substantially blocked Jurkat cell adhesion to ATX, whereas a function-blocking antibody to the β_2 integrin subunit and an irrelevant class-matched antibody had no effect (Fig. 4b). To determine whether a more physiological activator of integrins would increase Jurkat cell binding to ATX, we exposed lymphocytes to immobilized CCL21, an arrest chemokine^{3,7}. CCL21 stimulated increased adhesion of Jurkat cells to ATX and this interaction required $\alpha_4\beta_1$ (Fig. 4c). In addition, overnight treatment of Jurkat cells with the phorbol ester PMA resulted in increased $\alpha_4\beta_1$ -dependent Jurkat cell adhesion to ATX (not shown). Purified human T cells (isolated from peripheral blood) also bound to immobilized ATX. Mn^{2+} enhanced this interaction, as did co-immobilized CCL21 by about 2-fold. ATX was comparable to VCAM-1 in supporting T cell adhesion per unit input protein (Fig. 4d). Again, antibody blockade indicated the involvement of $\alpha_4\beta_1$ in the CCL21 and Mn^{2+} enhanced interactions, although inhibition by α_4 and β_1 antibodies was not as strong as with Jurkat cells (Fig. 4e,f), possibly because T cells express a lower amount of $\alpha_4\beta_1$ 27. Surprisingly, even though mouse lymphocytes displayed marked binding (EDTA-inhibitable) to immobilized ATX, we were not able to block this interaction with integrin-directed antibodies (specific for β_1 , β_2 or α_4) or with integrin-binding peptides (LDV and RGD) (Supplementary Fig. 3 online). Combinations of these antibodies were also ineffective.

LPA effects on lymphocytes

A major enzymatic product of ATX is LPA. Since ATX is capable of binding to lymphocytes (Fig. 4) and its substrate LPC is abundant in blood plasma¹³, we next explored the effects of LPA on lymphocytes. LPA has been reported to stimulate actin cytoskeletal reorganization in T cell lymphoma cell lines²⁸. We employed a fluorescent conjugate of phalloidin to measure the effects of LPA on actin polymerization in purified human T cells. LPA (5 μ M) induced a 40% increase in intracellular filamentous actin within 15 minutes of its addition (Fig. 5a). This increase was comparable to that induced in eosinophils by the same concentration of LPA²⁹. In agreement with previous work³⁰, CXCL12, a highly active chemokine for lymphocytes, also elicited actin polymerization in the T cells (Fig. 5a).

LPA is capable of promoting the invasiveness of T cell lymphomas into fibroblast monolayers²⁸. Additionally in a transwell assay, LPA was found to induce both chemotaxis and chemokinesis of Jurkat T cells, which were engineered to express predominantly a single

GPCR receptor for LPA (LPA₂)³¹. To determine whether LPA had motility-enhancing effects on primary human T cells, we carried out a conventional transwell assay. When LPA was added to the top well with lymphocytes, it stimulated migration to the lower well with strongest effect at 1 μ M (Fig. 5b). This chemokinetic activity was inhibited by pretreating the lymphocytes with pertussis toxin, consistent with the involvement of a G_i family G-protein (Fig. 5c). To test for chemotactic activity, we added LPA to the lower well alone. There was no effect on migration of the lymphocytes (Fig. 5d). Also, the addition of LPA at the same concentration to both wells produced the same effect as addition of LPA to the upper well, consistent with a chemokinetic action of LPA. The inability of LPA in the lower chamber to induce responses from lymphocytes may reflect the presence of very low levels of LPA in the upper chamber because of its non-specific binding to the plastic filter³¹. When a known chemotactic factor, CXCL12 or CCL21, was added to the lower well, its effects were additive with those of LPA. Thus with LPA in the upper well and CXCL12 or CCL21 in the lower well, migration to the lower well was greater than with LPA alone in the upper well or chemoattractant alone in the lower well (Fig. 5e,f). Parallel results were obtained with mouse lymphocytes (Supplementary Fig. 4 online). To determine whether LPA might be increasing receptor expression for CXCL12 or CCL21, we measured surface expression of CXCR4 and CCR7 on T cells after LPA treatment. There was no change in the amount of these receptors (Fig. 5g). We conclude that lymphocytes are able to respond simultaneously to the chemokinetic action of LPA and the chemotactic action of chemokines.

The effects of inactive ATX on T cell homing

The presence of receptor sites for ATX on activated lymphocytes together with the ability of LPA to stimulate lymphocyte migration suggested a potential function of ATX function during lymphocyte homing. In this paracrine model (Supplementary Fig. 5 online), ATX is secreted into the lumen of HEVs and binds to adherent lymphocytes through an interaction with an activated receptor ($\alpha_4\beta_1$ is shown as an example). ATX may first bind to HEVs and subsequently interact with lymphocytes as an immobilized ligand. Next, the lymphocyte-bound ATX acts on its abundant substrate LPC in the blood and catalyzes the production of a high local concentration of LPA, which triggers GPCRs on the lymphocyte and promotes transendothelial migration. This model predicts that the presence of an excess of catalytically inactive ATX in the blood could potentially act in a dominant-negative manner, by competing with endogenous ATX for a limited number of ATX-binding sites on the lymphocyte. To test this proposal, we prepared a mutant of human ATX (T210A) with a single amino acid change in the phosphodiesterase domain¹⁶, together with the wild-type protein (Supplementary Fig. 6 online). As expected, lysoPLD activity was absent in the mutant and readily detected in the wild-type protein. We showed that the T210A protein was equivalent to wild-type ATX in its ability to support the adhesion of Mn²⁺ stimulated lymphocytes (Supplementary Fig. 6 online). We tested the effects of these two proteins in a standard lymphocyte homing assay. Purified T lymphocytes were fluorescently labeled, mixed with inactive or active ATX or with PBS, and then injected intravenously into wild-type mice. After 15 minutes, the number of fluorescent cells that had accumulated in lymph nodes, Peyer's patches and spleen was determined by flow cytometry (Fig. 6a). Compared to PBS treatment, inactive ATX (4 μ g per animal) attenuated homing of T cells by 50–60% for lymph nodes and Peyer's patches and by 30% for spleen. Active ATX at the same concentration did not affect homing to any organ. The reduced accumulation of lymphocytes was not due to removal of lymphocytes from the blood, since treatment with inactive ATX had no effect on the number of labeled lymphocytes in the blood. Increasing the amount of inactive ATX to 8 μ g per animal did not augment inhibition (not shown).

To visualize the effects of ATX on the migration of lymphocytes into and within a lymphoid organ, we harvested lymph nodes 15 minutes after injection of labeled lymphocytes and

evaluated the number and position of fluorescent cells within sections. Consistent with the homing measurements, treatment of lymphocytes with mutant ATX (4 μ g per animal) decreased the total number of lymphocytes within the lymph sections by 50% relative to PBS treatment (Fig. 6b). To compare the effects of the two proteins on overall migration of the lymphocytes, we measured the position of individual cells relative to the nearest HEV (revealed by MECA-79 staining) and determined the distance migrated. Mutant ATX inhibited overall migration as compared to both the PBS and wild-type ATX treatments (Fig. 6c). Thus, the introduction of a low concentration of inactive ATX into the circulation caused a general reduction in lymphocyte homing to secondary lymphoid organs and impeded the dispersal of lymphocytes within at least one class of organ.

Discussion

Despite the fact that naive lymphocytes are intrinsically non-motile under standard *in vitro* conditions³², these cells constitutively enter lymphoid organs. Co-culture of lymphocytes with HECs promotes the efficient passage of lymphocytes across the endothelial layer, suggesting HECs express a 'lymphocyte migration stimulus'^{33,34}. Although a soluble factor, which may correspond to this activity has been described³⁴, the biochemical identity and mechanism of action of this 'stimulus' have not been defined. We report here that ATX is a strong candidate for the postulated lymphocyte motility stimulus.

Following up our initial gene profiling analysis of HECs¹⁰, we found that murine lymphoid organs expressed abundant transcripts for ATX and confirmed that HECs were a particularly rich source. The expression of ATX protein in lymph nodes HEVs was verified by immunocytochemistry and biochemical analysis of isolated HECs. We also found ATX expression in HEV-like vessels in two different models of lymphoid neogenesis. A number of proteins expressed by HEVs (including selectin ligand scaffolds, fucosyltransferases, sulfotransferases, and chemokines), which are critically involved in the lymphocyte homing cascade, are elements of an HEV differentiation program⁴. Lymphotoxin signaling is required for this program during organogenesis and for the induction of HEV-like vessels at sites of lymphoid neogenesis^{4,35}. Interestingly, *Enpp2* (which encodes ATX) is one of a limited subset of genes that is suppressed in lymph nodes by blockade of lymphotoxin signaling³⁶, suggesting that it may be an element of the HEV differentiation program.

Our finding that ATX was secreted by HECs in an apical orientation indicated a possible parallel with 'arrest' chemokines. These are secreted by HEVs, become associated with apical receptors, and exert their activities on adherent lymphocytes⁷. Noting a potential $\alpha_4\beta_1$ -binding motif (LDV) in the sequence of ATX, we asked whether ATX could bind to lymphocytes via $\alpha_4\beta_1$. Indeed, we found that Jurkat cells and primary human T cells, activated in three different ways, exhibited $\alpha_4\beta_1$ -dependent adhesion to immobilized ATX. This finding suggests that ATX could be targeted to HEV-adherent lymphocytes whose integrins have been activated during the arrest step of the recruitment cascade. A number of $\alpha_4\beta_1$ ligands are known, including fibronectin, VCAM-1 and osteopontin³⁷. ATX also possesses an RGD sequence, suggesting that ATX may be able to partner with other integrins²⁶. Further experiments are needed to define the nature of the divalent-cation dependent ATX receptors on murine lymphocytes. It is conceivable that the versatility of ATX may include the ability to bind simultaneously to both HEV and lymphocyte, which could help bridge the lymphocyte to the endothelium.

To arrive at a rationale for how ATX might influence lymphocyte behavior, we examined the effects of LPA on primary T cells. LPA was our focus because many of the biological activities of ATX are attributable to its production of this phospholipid¹³. Moreover, ATX accounts for the basal concentrations of LPA in the blood^{14,15}. Several previous reports had investigated the responses of lymphocytes (usually lymphoma populations) to LPA^{19,28,31,38–40}. Most

pertinent to the present study are the rapid actions of LPA in inducing chemokinesis of Jurkat cells³¹ and in promoting shape change and invasiveness of mouse lymphoma lines²⁸. Our experiments with primary lymphocytes are consistent with these previous cell line studies. Thus, we showed that LPA induced actin polymerization in suspended cells and was chemokinetic in a transwell assay. The dose-response curves were similar to those reported for LPA in other bioassays^{17,20,31}. Importantly, the optimal concentration for LPA in our experiments ($\approx 1 \mu\text{M}$) exceeds basal values for blood plasma both in mouse and human^{13–15}. Interestingly, we found that the chemokinetic effect of LPA was additive with the chemoattractant effects of CXCL12 and CCL21, suggesting the possibility of cooperative interactions between LPA and chemokine signaling in migrating lymphocytes.

Our strongest functional results came from short-term homing experiments. We showed that intravenous injection of an enzymatically-inactive form of ATX blocked homing of blood-borne lymphocytes to lymph nodes, spleen and Peyer's patches. Our model posits that the inactive ATX would interfere with the function of endogenous ATX by displacing it from a limited number of binding sites on lymphocytes (and possibly HECs), thus exerting a dominant negative effect. The endogenous ATX is proposed to emanate from HEVs. For spleen, the proposed sources are marginal zones, which surround white pulp regions and are known sites of lymphocyte entry. The net result of this competition from the inactive ATX would be reduced amounts of locally produced LPA and consequently a reduction in lymphocyte entry. Whether LPA is functioning to affect cell motility, cell adhesion (for example, post-arrest effects on LFA-1), cell shape, or transendothelial migration during the complex process of entry remains to be investigated. It should be noted that locally produced LPA at the lymphocyte-HEV nexus could also have effects on HEV function during lymphocyte recruitment, as LPA is capable of eliciting responses in endothelial cells⁴¹.

The activity of ATX in regulating lymphocyte migration may extend beyond the entry phase, since we detected ATX in stromal regions of lymphoid organs. Lymphocytes exhibit considerable motility within lymphoid organs, much of it apparently random in direction^{3,21,42,43}. CCR7 and its chemokine ligand CCL21 account for a portion of T cell motility, probably through a chemokinetic effect^{44–47}. LPA is a plausible candidate for an additional motility-stimulating factor within lymphoid organs. We found that treatment of mice with inactive ATX caused a reduction in the distance between labeled lymphocytes and the nearest HEVs. Whether this effect is attributable to delayed entry into the lymph node or reduced motility within the node is not known. With regard to a possible ATX contribution to lymphocyte egress from lymph nodes, we did not detect ATX in LYVE-1 positive vessels (not shown). Finally, it should be noted that ATX may be stably tethered to lymphoid stromal elements and thus could provide an adhesive substrate for migrating lymphocytes.

LPA signals cells through 5 known GPCRs (LPA_{1–5}), the first 3 of which are members of the EDG family to which the S1P receptors also belong^{19,48,49}. Lymphocytes predominantly express LPA₁^{40,48}, LPA₂^{38,48} and LPA₅⁴⁸ with subset preferences. Unlike chemokine GPCRs, which primarily signal through G_i family G-proteins²¹, LPA receptors can utilize a variety of G-protein families including the G_i family¹⁹. We found that the LPA-induced chemokinesis of lymphocytes was blocked by pertussis toxin, indicating the involvement of G_i proteins in this response. However, pertussis toxin has no effect on lymphoma invasiveness, whereas G-proteins of the G_q and G_{12/13} families are implicated²⁸. The LPA signaling pathways involved in lymphocyte migration into and within lymphoid organs remain to be studied.

Lymphocyte recruitment into lymphoid organs is a multistep process. Its molecular elucidation over the past 20 years represents a triumph of immunology. Here, we propose a novel step in this process involving the regulation of lymphocyte migration by the ectoenzyme ATX. Our

model of ATX action lends itself to testing with genetically engineered mice and pharmacological agents. As inhibitors of lymphocyte exit from lymphoid organs are of clinical value for achieving immunosuppression¹⁹, inhibitors of the proposed ATX pathway may also be of therapeutic value in some settings by preventing entry of lymphocytes into secondary lymphoid organs or into induced tertiary lymphoid organs at sites of chronic inflammation⁵⁰.

Methods

Human subjects

Peripheral venous blood was obtained from normal donors after informed consent under approval from Committee for Human Research at the University of California, San Francisco.

For some experiments, human T cells were prepared from peripheral blood purchased from the Blood Center of the Pacific, San Francisco.

Mice—All mouse experiments were performed in accordance with protocols approved by the Committee for Animal Research at the University of California, San Francisco. For real-time PCR measurements, C57BL/6 female mice (8 weeks) were used for RNA extraction. For homing assays, CD-1 female mice (6 to 8 weeks) were used for both recipient and donor mice. Mice were purchased from Charles River Laboratories.

Reagents, antibodies, and proteins

Fatty acid-free BSA, L- α -lysophosphatidic acid, L- α -lysophosphatidylcholine, choline oxidase, and peroxidase were obtained from Sigma. Immunocytochemistry was performed with a peptide polyclonal antibody. The antibody was prepared by immunizing rabbits with the peptide (CKKPDQHFHPYMKQHLPKRL) (ProSci Incorporated) and purifying the serum on a peptide affinity column as described⁵¹. The specificity of the antibody was verified by ELISA and peptide inhibition (Supplementary Fig. 1). ATX immunoblotting was performed with a previously reported ATX antibody directed to the same peptide sequence⁵¹. Other antibodies are specified in the sections below. Recombinant human and mouse VCAM-Ig were from R&D systems. Recombinant human CXCL12 and human CCL21 were from Leinco Technologies.

Cells

HECs were purified from mouse lymph nodes were prepared as described in previous reports^{9,10}. Mouse T cells were prepared from CD-1 mouse mesenteric and peripheral LNs. Non-T cells were depleted using mouse and human pan-T cell purification kits (Miltenyi Biotec). At least 98% of the resulting cells expressed CD3. Jurkat T cells were maintained in RPMI-1640 (Mediatech) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, 100 mcg/ml streptomycin, and 25 μ M 2-mercaptoethanol.

Cloning of human *ENPP2* cDNA and expression of ATX protein

A full-length *ENPP2* cDNA was cloned from a human tonsil HEC library⁵² using a pool selection technique in conjunction with PCR, based on previously described procedures⁵². The cDNA was sequenced in both directions and exactly matched the teratocarcinoma form of human ATX, which differs from melanoma ATX in lacking a 52 aa insertion. Native ATX produced by melanoma cells has an amino-terminus beginning at residue 49¹². A cDNA encoding a 51 residue N-terminal truncated version of ATX was cloned into the pSecTag expression vector (Invitrogen) with a 6 His tag at the amino terminus. This protein is referred to as WT ATX. We also prepared a cDNA encoding enzymatically inactive ATX (T210A), in which the critical amino acid residue Thr-210 was replaced with Ala¹⁶ by PCR-based

mutagenesis. The proteins were purified from supernatants of transfected COS-7 cells by chromatography on nickel-NTA sepharose (Qiagen). The purified proteins showed a single band in both gel staining (GelCode, Pierce) and immunoblotting after SDS-PAGE. Protein concentration was determined by the Bradford assay with BSA as the standard.

ATX secretion assay

To analyze ATX secreted from purified HECs, conditioned medium (CM) was prepared by culturing HECs in Opti-MEM I (Invitrogen) for 96 h with concentration by Vivaspin (Vivascience). CM proteins (2 µg/lane) were separated by SDS-PAGE (4–20% gradient gel, Bio-Rad) under reduction and transferred to a membrane (ProBlott, Applied Biosystems). After blockade with 3% BSA in PBS, the filter was probed with the ATX antibody or with a GlcCAM-1 antibody⁵³ followed by biotin-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) and streptavidin-horseradish peroxidase (Jackson ImmunoResearch) with detection (GE healthcare). Stable ATX transfectants of MDCK cells were prepared by transfecting with the pSecTag-hATX via Fugene6 (Roche) with selection in 0.5 mg/ml of zeocin (Invitrogen). The MDCK cells were cultured in transwell chambers (Corning, pore size 0.4 µm) for 4 days to allow confluent monolayers to form. Medium was replaced with Opti-MEM I and the cells were cultured for an additional 4 days. The cell culture supernatants from upper and lower chambers were analyzed for lysoPLD activity assay and were subjected to SDS-PAGE and immunoblotting. Antibodies to penta-His and clusterin (Santa Cruz) were used as primary Abs followed by HRP-conjugated secondary antibodies with detection by ECL. The membrane used for anti-penta His mAb was stripped and re-probed with mouse IgG.

Real-time PCR

Total RNA from each of 3 8-week-old female C57BL/6 mice tissue was extracted by Trizol (Invitrogen) and subjected to reverse transcription reactions with superscript II reverse transcriptase (Invitrogen) and random hexamers (Invitrogen). Real-time PCR was performed in the Biomolecular Resource Center at University of California San Francisco using TaqMan 7900HT (Applied biosystems). For measuring ATX mRNA expression, the following primer set and probe were used: forward primer, 5'-TGTGGAAGGCAGCTCTATTCTGT-3'; reverse primer, 5'-ATTGTCAGGTCGGTGAG GAAGGAT-3' probe, 5'-(FAM)-ACTTCACTCAGCCTGCAGACAAGTGT -(BHQ)-3'. PCR was performed at 95 °C 10 min, 40 cycles at 95 °C for 15 sec and 60 °C for 2 min, and analysis was carried out using SDS 2.1 software. The expression of ATX was normalized to hypoxanthine phosphoribosyltransferase (HPRT).

In situ hybridization

Peripheral lymph node paraffin sections (5 µm) from C57BL/6 mice were deparaffinized, fixed in 4% paraformaldehyde, and treated with proteinase K. After washing in saline citrate buffer, the sections were incubated with hybridization solution overnight with sense or antisense ³⁵S-labeled ATX riboprobe⁵². After hybridization, sections were washed, dipped in photographic emulsion NTB2 (Kodak), developed, and counterstained with hematoxylin and eosin.

Immunofluorescence

Mouse tissues were embedded and frozen in OCT compound. 10 µm thick cryostat sections were fixed in acetone, blocked with 3% BSA in PBS containing 10% goat serum and then stained with anti-ATX as a primary antibody followed by biotin conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and Cy2-streptavidin (Jackson ImmunoResearch Laboratories). For simultaneous MECA-79 staining, Cy3 goat anti-rat IgM (Jackson ImmunoResearch Laboratories) was used as a secondary detection antibody. Digital images

were captured with an Optiphot microscope (Nikon) equipped with a digital camera system (AxioCam; Zeiss).

LysoPLD assay

LysoPLD activity was measured as described¹⁷. In brief, ATX protein was incubated with 1 mM LPC at 37 °C for up to 4 h and liberated choline was determined colorimetrically employing linked reactions with choline oxidase (2 U/ml) and peroxidase (5 U/ml).

Static ATX adhesion assay

Wells of 96-well Immulon 2HB (Thermo) plate were coated with ATX in magnesium and calcium-free PBS overnight at 4°C. After rinsing with PBS, free binding sites were blocked with 0.2% polyvinylpyrrolidone (PVP; molecular weight 360 kDa; Sigma) for 2 h at 22°C.

When manganese was used for the assay, Jurkat T or human peripheral blood T cells labeled with 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen) were pretreated at 22°C for 30 min with 5 µg/ml of the indicated antibody in Hanks' balanced salt solution containing 25 mM HEPES pH 7.4, 0.5 mM manganese and 0.5% of BSA (fatty acid-free). For CCL21 stimulation, ATX (200 ng) and CCL21 (100 ng) were co-immobilized on the wells as described above. The cells were then added to the wells and allowed to settle for 1 h at 37 °C. After the nonadherent cells were removed by inverting the plate, the adherent cells were solubilized with 0.2% Nonidet P-40 in PBS, and fluorescence of the wells was measured with a Cytofluor II (Perseptive Biosystems). The following function-blocking antibodies were used: ICRF44 (α_M subunit, BioLegend), HP2/1 (α_4 subunit, Chemicon), P5D2 (β_1 subunit, Santa Cruz) and TS1/18 (β_2 subunit, BioLegend).

Transwell assay

Human T cells (10^6) were added to the upper chambers of Transwells (Costar, 5 µm pore size) and allowed to migrate for 3 h at 37 °C. To determine the chemokinetic response of T cells, the cells were incubated with various concentration of LPA in the upper chamber. In some experiments, CXCL12 or CCL21 was simultaneously added to the lower chambers as indicated in figure legends. For PTX inhibition experiments, T cells were preincubated with 250 ng/ml of PTX (Fluka) for 2–3 h at 37 °C. Migrated cells were quantified by flow cytometry.

F-actin reorganization assay

Purified T cells were incubated with or without LPA for 15 min at 37 °C. The cells were fixed and permeabilized with PBS containing 2% paraformaldehyde and 0.2% Triton X-100 and stained with Alexa 488-conjugated phalloidin (Invitrogen). The incorporation of fluorescent phalloidin into T cells was analyzed by flow cytometry.

Chemokine receptor expression

T cell chemokine receptor expression was examined by flow cytometry. After T cells were treated with LPA at 37 °C for 3 h, cells were incubated with anti-CXCL12 (BioLegend), anti-CCL7 (eBioscience) or isotype-matched control IgGs. The cells were incubated with phycoerythrin-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and analyzed by flow cytometry.

In vivo T cell homing assay

Purified T cells from peripheral and mesenteric lymph nodes of 6- to 8-week-old CD-1 mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) at 37 °C for 30 min. The cells were incubated with ATX protein (WT or inactive form, 40 µg/ml) or with PBS at 37 °C for 2 h. 5×10^7 cells in 100 µl of PBS along with the ATX protein (total of

4 μ g) were injected into age-matched CD-1 recipient mice via the tail vein. 15 min later, the mice were sacrificed and MLN, PLN, PP, spleen suspensions were prepared by mechanical dissociation. Blood samples were also collected. The number of the CFSE-positive cells in each sample (per 10^6 total lymphocytes for MLN, PLN, Spl and blood, 5×10^5 total cells for PP) were quantified by flow cytometry. Data were acquired by CellQuest (BD Bioscience) and analyzed with FlowJo (Tree Star). To determine the number and distribution of injected lymphocytes in lymph nodes, frozen blocks were prepared after sacrificing the animals, and 10 μ m sections were prepared and fixed with 4% PFA. The number of fluorescent cells in each field was quantified from 10 nonsequential sections. To identify HEV, sections were stained with biotin conjugated MECA-79 followed by streptavidin-HRP and NovaRED (Vector). To analyze the distance between fluorescent cells and HEVs, fluorescent cells outside of HEVs were identified and the distance to the center to the nearest HEV was measured for least 10 different nonsequential sections. The measurements were performed with the investigator blinded as to sample identity.

Statistical analysis

The Student t-test was used for statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used in this paper

HEV, high endothelial venule; HEC, high endothelial venule cell, lysoPLD, lysophospholipase D; MLN, mesenteric lymph node; PLN, peripheral lymph node; SPL, spleen; LPA, lysophosphatidic acid; ATX, autotaxin.

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Figure 1.

Expression of ATX transcripts in mouse tissues. **(a)** First strand cDNA was synthesized from RNA obtained from mouse tissues and subjected to quantitative PCR analysis. The abundance of ATX mRNA in each tissue was normalized to HPRT mRNA, and related to the normalized level in mesenteric lymph node (MLN). The tissues (or cell lines) analyzed were: PLN, peripheral lymph node; PP, Peyer's patch; HECs, high endothelial cells; SPL, spleen; THY, thymus; SC, spinal cord; MG, mammary gland; SI, small intestine; BM, bone marrow; bEnd. 3, mouse endothelial cell line; SVEC, mouse endothelial cell line. The data represent means and SD's from measurements performed on 3 independent animals. **(b)** ATX mRNA expression in PLN and spleen was determined by *in situ* hybridization using an ^{35}S -labeled

mouse ATX antisense probe. Specific signals were not present in the sense controls (not shown). These results are representative of two separate experiments. In both experiments at least one lymphoid organ of each type was examined at three exposure times. Bar denotes 100 μm . RP, red pulp; WP, white pulp.

Figure 2. Localization of ATX protein in lymphoid organs. Two-color immunofluorescence was performed on cryostat sections of PLN and MLN for ATX (green) and MECA-79 reactivity (red) and on spleen sections for ATX (green) and CD31 (red). Bright field images of the same fields (hematoxylin staining) are presented on the right. Scale bars, 100 μ m. White arrows indicate ATX-positive small vessels, which are negative for MECA-79. The results shown are representative of 4 independent experiments.

Figure 3.

Secretion of ATX and HECs and transfected MDCK cells. **(a)** Conditioned medium from isolated HECs was subjected to SDS-PAGE and immunoblotted for ATX and GlyCAM-1. The membrane was stripped and reprobed with normal rabbit IgG as a control. The results shown are representative of three independent experiments. **(b)** MDCK monolayers (ATX-transfected or mock-transfected) were evaluated for integrity by measuring diffusion of FITC-dextran across the monolayers in transwell units. Diffusion across a bare filter is shown for comparison. **(c,d)** Supernatants were collected from above (apical) or below (basal) the MDCK monolayers. In **c**, the supernatants were immunoblotted for ATX or clusterin (Clstr) or with control mouse IgG. In **d**, the supernatants were analyzed for ATX enzymatic activity (lysoPLD). The slight

difference in molecular weight between rATX and native ATX in **a** is likely due to glycosylation differences. The results in **b**, **c** and **d** are representative of two independent experiments.

Figure 4a, 4b and 4c

Figure 4d, 4e and 4f

Figure 4.

Integrin dependency of ATX binding to human T cells. **(a)** The interaction of ATX with Jurkat T cells was examined by a static adhesion assay using ATX-coated plastic wells (at the indicated input concentration) in divalent cation-free Hanks' balanced salt solution with or without 0.5 mM Mn^{2+} . sICAM and sVCAM (human, 200 ng) were coated onto wells to serve as positive controls for adhesive substrates. Results are shown as means $\pm$ SD's. of triplicate determinations. **(b)** Jurkat T cells were pretreated with 5 μ g/ml of function-blocking antibodies for integrin α_M , α_4 , β_1 or β_2 in Mn^{2+} containing buffer and then tested for adhesion to ATX (200 ng) in the presence of Mn^{2+} . Two separate experiments are presented in the left and right panels. Adhesion to VCAM-1-coated wells (200 ng) is shown for comparison. Results are

presented as means $\pm$ SD's. of triplicate determinations. **(c)** The interaction of ATX with CCL21-stimulated Jurkat T cells was examined by static adhesion assay. The cells were allowed to interact with CCL21 (100 ng) co-immobilized with ATX protein (200 ng) on the plate. Results are shown as means $\pm$ SD's of triplicate determinations. **(d)** Human T cell binding was measured to ATX- or VCAM-1-coated wells at the indicated substrate concentration in the presence of Mn^{2+} . Results are shown as means $\pm$ SD's. of triplicate determinations. **(e)** Function-blocking antibodies against integrin subunits were tested for their effects on the adhesion of human T-cells to wells coated with ATX (200 ng) in the presence of Mn^{2+} . Adhesion to VCAM-1 (200 ng) is shown for comparison. Results are presented as means $\pm$ SD's. of triplicate determinations. **(f)** ATX interaction with primary human T cells. Human T cells were exposed to co-immobilized CCL21 and ATX as above. Adhesion was tested after treating the cells with function-blocking antibodies as indicated. Results are shown as means $\pm$ SD's of triplicate determinations. * denotes that the inhibitory effect of the antibody relative to the control was significant with a *P* value of < 0.01 . The results in **a–c** are representative of 3–4 and those of **d** and **f** 2 experiments. The experiment presented in **e** was performed once.

Figure 5.

LPA effects on human T cells. **(a)** T cells were stimulated by the indicated amount of LPA or CXCL12 at 250 ng/ml for 37 °C for 15 min, fixed and permeabilized and then stained with Alexa 488-conjugated phalloidin. The amount of F-actin is shown normalized to that in untreated cells. **(b)** T cells were added to the upper chamber of transwell units with LPA added either to the same chamber (to induce chemokinesis) or to the lower chamber (to induce chemotaxis). After 3 h of incubation at 37 °C, cells that had migrated to the bottom chamber were quantified by flow cytometry. CXCL12 (250 ng/ml in the lower chamber) was used as a positive control for a lymphocyte chemoattractant. **(c)** T cells were either pretreated with PTX or not and then tested for their chemokinetic response to LPA in same assay as above. **(d)** LPA

was added at 1 μ M to the upper, lower, or to both chambers and tested for its effects on T cell migration from the upper to lower chamber after 3 h. **(e)** The transwell assay was performed as above with LPA in the upper chamber (1 μ M) and/or CXCL12 (50 ng/ml) in the lower chamber. In all experiments, results are presented as means $\pm$ SD's of triplicate determinations. **(f)** Human T cell migration assay was performed with 1 μ M of LPA in the upper chamber (1 μ M) and/or CCL21 (50 ng/ml) in the lower chamber. In all experiments **(a–f)**, results are presented as means $\pm$ SD's of triplicate determinations. The * indicates that the combination of chemokine (CXCL12 or CCL21) and LPA induced more T cell migration than chemokine alone ($P < 0.01$). **(g)** Chemokine receptor expression of LPA-stimulated T cells was tested by flow cytometry. T cells were incubated with indicated concentration of LPA at 37 °C for 3 h. The cell surface receptors were detected with anti-CXCR4 or anti-CCR7 mAb. The results in **a, b, and g** are representative of two independent experiments. The experiments in **c, d, e, and f** were each performed once.

a

b

c

Figure 6.

Migration of T cells to and within lymphoid organs in the presence of exogenous ATX. **(a)** CFSE-labeled T cells were pretreated with PBS, active ATX (WT) or inactive ATX (T210A) and the mixtures were injected intravenously into recipient mice. The amount of ATX was 4 μ g per animal. 15 min later, mice were sacrificed and fluorescent cells in each tissue were quantified by flow cytometry. The relative homing indexes are shown as means $\pm$ SD's with results pooled from two experiments. For the lymphoid organs, $n = 6$ for PBS, and $n = 8$ for both WT and inactive ATX. For blood, $n = 4$ for each condition. * signify P values of <0.01 as compared to PBS controls. The results are representative of 5 experiments. **(b)** The entry of lymphocytes into PLNs was independently determined by counting the number of fluorescent

lymphocytes present in cryostat sections. As above, lymph nodes were processed 15 min after injection of labeled lymphocytes. Total lymphocytes were counted in at least 10 non-sequential sections for each treatment with three animals per treatment (PBS, WT ATX or T210 ATX). The results are shown as means $\pm$ SD's and are representative of two independent experiments. (c) Lymphocyte migration within PLNs was determined by measuring the distance between extravasated lymphocytes (outside of HEVs) and the nearest HEV. With the same treatments as above, lymph nodes were processed 15 min after injection of fluorescent lymphocytes. HEVs were identified by staining with MECA-79. The upper three panels show representative sections for the three treatments. The bottom graph shows all of the individual measurements taken from 10 non-sequential sections from 3 independent animals per treatment. The data are representative of two independent experiments. * denotes a *P* value of <0.05