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Autotaxin, LPA Receptors (1 and 5) Exert Disparate Functions in Tumor Cells Versus the Host Tissue Microenvironment in Melanoma Invasion and Metastasis

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Abstract

Autotaxin (ENPP2/ATX) and lysophosphatidic acid (LPA) receptors represent two key players in regulating cancer progression. The present study sought to understand the mechanistic role of LPA G protein-coupled receptors (GPCR), not only in the tumor cells but also in stromal cells of the tumor microenvironment. B16F10 melanoma cells predominantly express LPA₅ and LPA₂ receptors but lack LPA₁. LPA dose-dependently inhibited invasion of cells across a Matrigel layer. RNAi-mediated knockdown of LPA₅ relieved the inhibitory effect of LPA on invasion without affecting basal invasion. This suggests that LPA₅ exerts an anti-invasive action in melanoma cells in response to LPA. In addition, both siRNA-mediated knockdown and pharmacological inhibition of LPA₂ reduced the basal rate invasion. Unexpectedly, when probing the role of this GPCR in host tissues, it was found that the incidence of melanoma-derived lung metastasis was greatly reduced in LPA₅ knockout (KO) mice compared to wild-type (WT) mice. LPA₁- but not LPA₂-KO mice also showed diminished melanoma-derived lung metastasis, suggesting that host LPA₁ and LPA₅ receptors play critical roles in the seeding of metastasis. The decrease in tumor cell residence in the lungs of LPA₁- and LPA₅-KO animals was apparent 24 h after injection. However, KO of LPA₁, LPA₂ or LPA₅ did not affect the subcutaneous growth of melanoma tumors.

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Implications—These findings suggest that tumor- and stromal-LPA receptors, in particular LPA₁ and LPA₅, play different roles in invasion and the seeding of metastasis.

Keywords

Autotaxin; Lysophosphatidic acid receptor; Invasion; Metastasis; Tumor microenvironment

Introduction

One of the first indications for the involvement of autotaxin (ATX) in cancer was the identification of this secretory protein in human melanoma cells capable of stimulating cancer cell motility (1). The mechanism by which ATX acts as a motogen remained elusive until it was established that ATX has lysophospholipase D activity and is the primary enzyme responsible for lysophosphatidic acid (LPA) production in biological fluids (2, 3). Notably, plasma LPA levels in heterozygous knockout (KO) mice for the ATX gene (*atx*^{+/-}) were reduced by half compared to levels in wild-type (WT) littermates (4, 5). Aberrantly high expression of ATX has been detected in breast cancer (6), glioblastoma multiforme (7), prostate cancer (8), hepatocellular carcinoma (9), and melanoma (10). Overexpression of ATX in these malignancies promotes tumor motility and invasiveness, enhances metastatic potential, and is commonly associated with poor clinical outcomes (11).

LPA appears to be responsible for many of ATX's biological activities. This is not surprising, as early studies have demonstrated that LPA has pro-tumorigenic effects on ovarian, breast, and prostate cancer cells (12, 13). High levels of LPA were also reportedly present in ascites of patients with ovarian cancer (14). In cases of follicular lymphoma and of pancreatic cancer, elevated levels of ATX were detected in sera of patients with a concomitant increase in plasma LPA levels (15, 16).

LPA exerts its cellular functions by acting on specific G protein-coupled receptors (GPCR). There are at least nine GPCR that were reported to be activated by LPA, amongst which LPA₁, LPA₂, and LPA₃ receptors have been extensively studied. LPA₁₋₃ receptors belong to the same endothelial differentiation gene (*edg*) GPCR subfamily as the sphingosine-1-phosphate receptors (17). Consistent with the reported roles of ATX and LPA in cancer, it seems apparent that LPA receptors are also involved in tumorigenesis. Indeed, compelling evidence shows that LPA receptors are overexpressed in most cancers (18, 19) and function to enhance cancer cell survival, motility, invasion, and metastasis (20-22). In particular, both LPA₁ and LPA₂ receptors are implicated in breast cancer progression; the LPA₁ receptor was found to promote the metastasis of human breast cancer xenografts to the bone (23), whereas LPA₂ receptor expression was elevated in the majority of postmenopausal breast cancers (24). In cases of ovarian cancer, abundant expression of LPA₂ receptors was associated with an increase in invasiveness (25) and lipogenesis of ovarian cancer cells (26). In addition, expression levels of LPA₂ receptors in colorectal cancer were reportedly high and further increased during malignant transformation (27). A role for the LPA₃ receptor in regulating growth and survival of metastatic melanoma has also been documented. However, it is important to note that not all metastatic melanomas express the LPA₃

receptor, as data from gene expression analyses of human melanoma specimens revealed high variability in expression profiles amongst samples (10).

In contrast to LPA₁₋₃ receptors, little is known about the roles of LPA₄ (GPR23/P2Y9), LPA₅ (GPR92), and LPA₆ (P2Y5) receptors in cancer (11); and characterization of the putative LPA GPCR GPR87, P2Y10, and GPR35 awaits further research. Only a few studies have been carried out to elucidate the function of LPA₄ in tumorigenesis. Specifically, work by Harper and colleagues demonstrated that the LPA₄ receptor was responsible for mediating ATX-induced invadopodia formation in fibrosarcoma cells, a critical step in the mesenchymal form of invasion (28). This report however, contradicts the findings from an earlier study, in which LPA₄ was found to inhibit cell motility and invasion of mouse embryonic fibroblasts (29). It has recently been shown that the LPA₅ receptor may potentially inhibit migration of B16 melanoma cells by activating the cAMP-PKA pathway and diminishing PIP3 signaling (30).

Although much interest has been shown in elucidating the functions of ATX and LPA receptors in cancer cells, the effect of stromal or host LPA receptors on tumor-microenvironment (TME) interactions has not been addressed. It is becoming clear that the TME plays a fundamental role not only in supporting tumor growth but also in its progression toward a metastatic disease. In fact, tumor cells are capable of establishing a reciprocal communication with their host microenvironment to create the most favorable environment for them to proliferate, migrate, invade, and metastasize (31). In the present study, we evaluated the function of the ATX-LPA receptor signaling axis in TME interaction, particularly in the homing of metastasizing cancer cells. The syngeneic B16 murine melanoma model was chosen as our platform for assessing tumor-stroma interactions, with good reasons. We have previously shown that the metastasizing capacity of B16F10 melanoma cells *in vivo* is in part attributable to ATX (32, 33). In addition, the availability of LPA receptor KO mice generated in a mixed C57BL/6 and 129/Sv genetic background allows the use of this model to study the role of host LPA receptors in metastasis. Our data demonstrate that the homing of metastasizing B16F10 melanoma cells to the lungs is substantially reduced by the absence of host LPA₁ and almost completely reduced by the absence of LPA₅, whereas LPA₂ and LPA₅ expressed in the tumor cell promote and inhibit invasion, respectively.

Materials and Methods

Materials

Lysophosphatidic acid (18:1) and lysophosphatidylcholine (18:1) were purchased from Avanti Polar Lipids (Alabaster, AL). ATX inhibitor BMP22 and specific LPA₂ antagonist compound 35 were synthesized as described in (32) and (34). Ki16425 was purchased from Cayman Chemical (Ann Arbor, MI). Stock solutions (10 mM) of BMP22, compound 35, and Ki16425 were prepared in dimethyl sulfoxide (DMSO) for *in vitro* studies. A stock solution of LPA (1 mM) was prepared as an equimolar complex with charcoal-stripped, fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich; St. Louis, MO) in phosphate-buffered saline (PBS). Polyethylene glycol 400 (PEG 400) was purchased from Sigma-Aldrich. All cell culture media were purchased from Corning Cellgro (Manassas, VA), and

cell culture reagents were from Life Technologies (Grand Island, NY) unless otherwise stated. The fluorescent ATX substrate FS-3 was purchased from Echelon Biosciences (Salt Lake City, UT).

Cell culture

B16F10 melanoma cells (gift from Dr. Gordon Mills, University of Texas, MD Anderson Cancer Center) were cultured in MEM supplemented with 5% heat-inactivated HyClone® FBS (Thermo Scientific; Pittsburgh, PA), 2 mM L-glutamine, 1X MEM vitamin, 1X MEM non-essential amino acid (NEAA), 1 mM sodium pyruvate, and 1X antibiotic-antimycotic. The highly invasive MM1 rat hepatoma suspension cells (gift from Dr. Michiko Mukai, Osaka University, Japan) were grown in DMEM supplemented with 10% (V/V) FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Human umbilical vein endothelial cells (HUVEC) and rat lung microvascular endothelial cells (RLMVEC) were purchased from VEC Technologies Inc. (Rensselaer, NY) and cultured in MCDB-131 complete medium supplemented with 10% (V/V) FBS, 90 µg/ml heparin, 10 ng/ml EGF, 1 µg/ml hydrocortisone, 0.2 mg/ml EndoGrowth, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 25 µg/ml amphotericin B. The isolation and culture of mesothelial cells from mice has been previously described elsewhere (35). Primary lung microvascular endothelial cells (MLVEC) from C57BL/6 mice were purchased from Cell Biologics, Inc. (Chicago, IL) and cultured in M1168 complete medium as described by the manufacturer's protocol.

Primary rat ATII cells were isolated according to the methods described previously (36, 37). Briefly, ATII cells were isolated from male Sprague-Dawley rats by elastase digestion and differential adherence on IgG-coated dishes. ATII cells were identified using Nile Red (Sigma-Aldrich) staining of lamellar bodies, and >95% of the cells were Nile Red-positive on *day 2*. Six-well plates that were coated with matrix deposited by rat lung fibroblasts (American Type Culture Collection; Manassas, VA) were used for culture of ATII cells. Freshly isolated cells were seeded to confluence at 3.5×10^6 cells/well in ATII culture medium (DMEM with 10% FBS, 4 mM glutamine, 1% penicillin/streptomycin, and 0.25 µM amphotericin B), and experiments were performed on *day 2* after isolation. To obtain ATI-like cells, ATII cells were cultured until *day 6* from the day of isolation, changing the media every day. On *day 2* or *day 6*, cells were harvested and total RNA was isolated using the RNA-isolation kit, RNeasy (Qiagen; Valencia, CA).

Short hairpin RNA (shRNA) knockdown in B16F10 melanoma cells

Five clones of lentiviral shRNA LPA₅ constructs (SHCLNG-XM_355812) were purchased from Sigma-Aldrich. Viral particles were produced by co-transfection of 293FT cells with an shRNA-expressing plasmid (CCGGCGTGCTGGATCCACTGGTTTACTCGAGTAAACCAGTGGATCCAGCACGTTTGTG), or pLK01 scramble, and a lentiviral packaging mix (Virapower) as described previously (38). B16F10 cells were plated onto a 35-mm dish at a density of 2×10^5 in growth medium, and incubated overnight. The following day, the cells were transduced with the lentiviral shRNA with 6 µg/ml polybrene in 1 ml of growth medium. One day after the transduction, the medium was replaced with fresh growth medium containing 1 µg/ml

puromycin (Sigma-Aldrich), and the cells were cultured for at least an additional week before performing functional studies.

Small interfering RNA (siRNA) knockdown in B16F10 melanoma cells

siRNA pools specific for LPA₂ and non-targeting scrambled siRNA were purchased from Dharmacon (GE Healthcare; Wauwatosa, WI). B16F10 cells were plated onto a 35-mm dish at a density of 2×10^5 in growth medium, and incubated overnight. The following day, the cells were transfected with 30pmol of siRNA using Lipofectamine® RNAiMAX reagent (Life Technologies; Grand Island, NY) according to the manufacturer's protocol. 24 hours after siRNA transfection, cells were harvested and used in the invasion assay.

Lentiviral overexpression of LPA₁ in B16F10 melanoma cells

B16F10 cells were transduced with enhanced green fluorescent protein (GFP), empty vector, or LPA₁-containing lentiviruses, respectively, in the presence of 6 µg/ml polybrene in 1 ml of growth medium. One day after transduction, the medium was replaced with fresh growth medium, and cells were selected with 1 µg/ml puromycin for one week.

RNA isolation and real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted using Trizol® reagent (Life Technologies; Grand Island, NY) followed by treatment with DNase I (Sigma-Aldrich) to remove any trace of genomic DNA contamination. Single-stranded cDNA was synthesized using the ThermoScript™ RT System (Life Technologies). Quantitative real-time PCR was performed using StepOnePlus™ instrument (Applied Biosystem) using the RT² RealTime™ SYBR Green/ROX PCR Master Mix Kit (Qiagen). The mRNA amplification cycle was as follows: 40 cycles of 95°C for 15 s and 60°C for 60 s. Each sample was normalized to the relative expression levels of GAPDH. Primer sequences are listed in Supplementary Table 1.

Measurement of ATX activity

Conditioned media (CM) from B16F10, MLVEC, MM1, or mesothelial cells (HUVEC or RLMVEC) were respectively prepared by incubating 5×10^6 cells for 20 h in serum-free MEM, M1168, DMEM, or MCDB-131. Control media were prepared from MEM, M1168, DMEM, or MCDB-131 without exposure to cells. Collected CM were centrifuged, filtered through a 0.22-µm filter, and concentrated (~25-fold) using Amicon Ultra 30 000 Centrifugal Filter Units (Millipore; Billerica, MA). ATX/lyso-PLD activity was measured by incubating 20µl of concentrated CM or mouse plasma with 2µM of the fluorogenic substrate FS-3 and 10µM of BSA. The change in fluorescence intensity was monitored for 3.5 h at 37°C using the FLEXStation II, with excitation and emission wavelengths of 485 nm and 538 nm, respectively. The differences in fluorescence intensity for each time point were normalized to that of time zero.

MM1 hepatoma cell invasion of endothelial monolayers

For *in vitro* tumor cell invasion of the endothelial barrier, 1.3×10^5 HUVEC (passages 4-6) were seeded into each well of a 12-well plate pre-coated with 0.2% gelatin (Sigma-Aldrich) and incubated for 2 d to form a confluent monolayer. Mesothelial cells harvested from three

mice were initially plated in 6-well plates pre-coated with poly-L-lysine (Sigma-Aldrich) and grown to confluence in DMEM supplemented with 10% (V/V) FBS in the presence of 2 mM L-glutamine, 100 U/ml penicillin G, and 10 µg/ml streptomycin. When confluent, 1.8×10^5 mesothelial cells were plated into each well of a 12-well plate and grown to confluence. For all invasion assays, MM1 cells were pre-stained with 2 µg/ml calcein AM (Invitrogen; Grand Island, NY) for 2 h, rinsed once, and seeded at a density of 5×10^4 cells per well over the monolayers. Tumor cells were left to invade the monolayers for 24 h either in serum-free MCDB-131 media (for HUVEC monolayer) or DMEM (for mesothelial monolayer) supplemented with 0.1% fatty acid free BSA. The day after MM1 cell seeding, non-invaded tumor cells were removed by repeated rinses of the monolayer with PBS (containing Ca^{2+} and Mg^{2+}), followed by fixation with 10% buffered formalin. The number of tumor cells that penetrated the monolayer was photographed under a NIKON TiU inverted microscope using phase-contrast and fluorescence illumination in a minimum of five non-overlapping fields at 100x magnification. The fluorescent images were overlaid on top of the phase contrast images using Elements BR software (version 3.1x), and the invaded MM1 cells showing the characteristic flattened morphology underneath the monolayer were counted.

Boyden chamber invasion of B16 melanoma cells

Cell invasion across a Matrigel-coated membrane was performed using the 8-µm pore size, 24-well BD Biocoat™ tumor invasion system (BD Biosciences; San Jose, CA). The Matrigel coating of the plates was rehydrated with PBS for 2 h at 37°C according to the manufacturer's protocol. The PBS was removed, and 1×10^5 cells in serum-free MEM supplemented with 0.1% BSA were added to each upper chamber; 0.75 ml of serum-free MEM/0.1% BSA containing LPA was added to the bottom chamber as a chemoattractant. In experiments with compound 35 or BMP22 respectively, the compounds were added to both the top and bottom chambers. Cells were left to invade the Matrigel for 20 h at 37°C. After incubation, the medium in the upper chamber was removed, and the insert was transferred into a new 24-well plate containing 4 µg/ml of calcein AM in Hank's balanced salt solution (HBSS). The plates were incubated for 1 h at 37°C. The fluorescence of invaded cells was then measured with a FLEXStation II plate reader at excitation and emission wavelengths of 485 and 530 nm, respectively.

B16F10 lung experimental metastasis model

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee and were consistent with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, revised 1985). B16F10 cells (1×10^5) were injected into the various LPA receptor KO female mice and their respective female WT littermates via tail vein. All mice were sacrificed at *day 21*. The lungs were harvested and inflated, and the number of metastatic tumor nodules was counted. Necropsy was also conducted to detect extrapulmonary metastases.

Where indicated, 1×10^5 B16F10 cells transduced with either lentivirus containing scrambled shRNA or shRNA for LPA₅ were injected into the tail vein of 8- to 12-wk-old female C57BL/6 mice (purchased from The Jackson Laboratory; Bar Harbor, Maine). In experiments involving treatment with BMP22 and/or Ki16425, 1×10^5 B16F10 cells were

injected into the tail vein of 8- to 12-wk-old female C57BL/6 mice. Thirty minutes later, each group of mice received treatment with vehicle (PBS with 10% PEG), or Ki16425 (1.5 mg/kg⁻¹), or BMP22 (0.1 mg/kg⁻¹), or a combination of Ki16425 and BMP22 via intraperitoneal injection daily for 20 days.

Histopathological examination

Lungs were excised from mice on *day 21* and fixed in 10% buffered formalin. Multiple paraffin-embedded 5-μm sections of the lungs were stained with H&E.

Lung extravasation assay

In some experiments, 1×10^6 B16F10 cells stably expressing GFP were injected via tail vein into the various LPA receptor KO female mice and their respective WT littermates. Mice were sacrificed 6 h and 24 h after injection. Lungs were harvested and filled with PBS, and the GFP-expressing B16F10 cells on the lung surface were imaged using a Nikon TiU inverted fluorescence microscope (using a low-magnitude 4X objective lens). At least 12 images were taken for each mouse, and tumor burden was measured as total area of GFP fluorescence (in pixel units) using the Nikon NIS element software.

Subcutaneous inoculation of B16F10 cells

For subcutaneous tumor growth, 4×10^5 B16F10 cells were injected subcutaneously into the left flank of each mouse in a 100-μl volume. The cells were prepared by reconstituting with Extracel-X® Hydrogel (Glycosan BioTime, Inc.; Alameda, CA) in a 1:1 dilution with culture medium as recommended by the manufacturer's protocol. All mice were sacrificed on *day 10*, and tumors were measured using a caliper, harvested, and weighed. Tumor volume was calculated using the modified ellipsoid formula: Tumor volume = $1/2$ (length $\times$ width²), where length is the greatest longitudinal diameter.

Statistical analysis

All statistical analysis were performed with GraphPad Prism software (version 5.0) using the two-tailed unpaired Student's *t*-test or the one-way ANOVA, followed by either a Bonferroni post-test or Newman-Keuls multiple comparison test.

Results

Differential expression of ATX and LPA receptors in tumor and stromal cells

To understand the roles of ATX and LPA receptors in TME interaction, we first sought to determine their expression profiles in tumor and stromal cells. Quantitative real-time PCR was performed on mRNA isolated from B16F10 melanoma cells and C57BL/6 lung microvascular endothelial cells (MLVEC). We found that B16F10 cells showed robust expression of transcripts encoding ATX, LPA₅, LPA₂, and LPA₆ receptors (Fig. 1A). In contrast, MLVEC had very low expression of ATX and LPA₅ transcripts and predominantly expressed LPA₆, LPA₁, and LPA₄ (Fig. 1B). We observed a similar trend in our previous studies, in which expression of ATX was higher in MM1 rat hepatocarcinoma cells compared to expression in stromal cells such as HUVEC and isolated murine mesothelial

cells (32). Because ATX is primarily a secreted enzyme that hydrolyzes lysophosphatidylcholine (LPC) to LPA, we decided to measure ATX activity in concentrated CM of B16F10 cells and MLVEC. To do this, we utilized the synthetic fluorogenic substrate FS-3, which was cleaved by ATX to release the fluorescent moiety from the quencher (39). Consistent with the ATX expression profiles in these cells, a higher level of ATX activity was detected in CM from B16F10 cells but not in CM from MLVEC (Fig. 1C and D). We extended this assessment to include concentrated CM from MM1 hepatocarcinoma, HUVEC, rat lung microvascular endothelial cells (RLMVEC), and mesothelial cells. Except in MM1 cells, we were unable to detect ATX activity in the CM from any of these stromal cells (Supplementary Fig. 1). On the basis of these observations, we concluded that B16F10 cells may provide a rich source of ATX within the tumor microenvironment.

LPA₅ receptor in B16F10 cells inhibits cell invasion *in vitro*

We have previously determined that ATX is involved in the formation of B16F10 lung metastases in C57BL/6 mice (32, 33). To determine whether LPA receptors contribute to the invasive behavior of these cells, Boyden chamber assays were conducted. We observed that B16F10 cells had a high basal invasion rate across the Matrigel layer; but when LPA was added to the bottom chamber as a chemoattractant, cell invasion was reduced (data not shown). To determine which LPA receptor was responsible for this effect, we used a lentiviral system to deliver an shRNA construct to knock down the LPA₅ receptor, the predominant LPA receptor expressed in B16F10 cells. This system effectively reduced the levels of LPA₅ mRNA by 80% compared to the levels in B16F10 cells transduced with scrambled shRNA (Fig. 2A). We found that knockdown of LPA₅ in B16F10 cells moderately attenuated the inhibitory effect of LPA on invasion. This effect was observed only when concentrations higher than 30 nM LPA were used (Fig. 2B). In addition, the basal invasion rate of B16F10 cells was unaffected by the knockdown of LPA₅.

We next examined the role of the LPA₂ receptor in B16F10 cell invasion. To do this, we utilized a siRNA targeting system to knockdown LPA₂ in B16F10 by 75% (Fig. 2C) and assessed the effect on invasion. We found that knockdown of LPA₂ significantly reduced the basal invasion rate of B16F10 compared to the scrambled siRNA control (Fig. 2D). To complement this finding, we used a potent and selective small-molecule antagonist for LPA₂, designated as compound 35 by Beck et al. (34). Consistent with the siRNA knockdown results, treatment with compound 35 alone dose-dependently inhibited the basal invasion rate of B16F10 across the Matrigel layer. These results suggest that LPA₂ may be, in part, responsible for the high basal invasion rate observed in B16F10 cells (Fig. 2E).

To further validate these results, we transduced B16F10 cells, which do not express LPA₁ mRNA (Fig. 1A), with an LPA₁ construct using the lentiviral system (Fig. 2F). We hypothesized that introducing the pro-invasive LPA₁ receptor might affect the outcome of invasion in these cells. Indeed, we noted that the invasion rate of LPA₁-overexpressing B16F10 cells in response to LPA increased significantly compared to the rate of vector-transduced B16F10 cells (Fig. 2G). In fact, cell invasion was greatest at 10 nM of LPA, and it decreased in magnitude at higher concentrations of LPA. We reasoned that this might be

attributed to the preference of LPA₁ receptors for the acyl form of LPA (18:1) used in the assay, whereas the more preferred ligand for the LPA₅ receptors is alkyl-glycerophosphate (AGP, also known as alkyl-LPA) (40).

Although these findings seemingly suggest that LPA₅ acts as an anti-invasive receptor in B16F10 cells *in vitro*, we found it difficult to reconcile them with the high metastatic capacity of these cells when injected into mice. Hence, we assessed the effect of shLPA₅ knockdown in B16F10 cells on the formation of lung metastases in C57BL/6 mice. Interestingly, we observed no significant difference in the number of lung metastases formed in mice inoculated with either shLPA₅ or scrambled shRNA B16F10 cells (Fig. 2H).

Host LPA₁ and LPA₅ receptors affect the homing of metastasizing B16F10 cells *in vitro*

Next, we investigated whether host LPA receptors play a role in influencing the homing of metastasizing tumor cells. For this purpose, we utilized a transcellular invasion model described previously by Mukai and colleagues (41). This model measures the invasion of MM1 rat hepatocarcinoma cells across a mesothelial monolayer that commonly lines the body's serous cavities. We isolated mesothelial cells from LPA₁-, LPA₂-, and LPA₅-KO mice and subjected the cultured monolayers to invasion by tumor cells. We found that the rate of MM1 invasion was significantly reduced in mesothelial monolayers generated from LPA₁- but not from LPA₂- and LPA₅-KO mice (Fig. 3A). This was the first indication of a possible involvement of stromal LPA₁ receptors in tumor invasion. To confirm this finding, we applied the LPA_{1/3} receptor antagonist Ki16425 to co-cultures of MM1 cells and HUVEC monolayers, and quantified invasion after 24 h. Ki16425 dose-dependently inhibited the LPA-induced MM1 invasion across the HUVEC monolayer, thus reinforcing our earlier observations in the LPA₁ KO mesothelium (Fig. 3B).

To complement our *in vitro* observations, we tested the effect of Ki16425 in the B16 experimental metastasis model. Briefly, 1×10^5 B16F10 cells were injected into the tail vein of female C57BL/6 mice. The mice were treated daily with Ki16425, and lungs were harvested on *day 21* for quantification of metastases. As a positive control, a parallel group of mice was treated with BMP22, an ATX inhibitor that was characterized recently by our group as being effective in reducing the metastasis of B16F10 cells to the lungs (32). In this model, administering Ki16425 significantly reduced the number of metastatic nodules formed in mice. In this regard, we found Ki16425 to be as effective as BMP22 in reducing the number of metastatic foci (Fig. 3C). Next, we questioned whether metastasis could be further reduced by concurrent administration of Ki16425 and BMP22. Our data showed that combination therapy was more effective in reducing the number of lung tumor nodules than single therapy with either compound (Fig. 3C).

Because B16F10 cells do not express LPA₁ receptors, we postulated that the effect of Ki16425 in reducing lung metastasis might be due to the inhibition of host LPA₁ receptors. To further validate this hypothesis, we injected B16F10 cells into LPA₁ KO mice and examined lung metastasis on *day 21*. Consistent with the data obtained using Ki16425, LPA₁ KO mice had significantly fewer metastatic nodules compared to WT mice (Fig. 4A and B). We extended these studies to include LPA₂- and LPA₅-KO mice. Interestingly, we found that deletion of the host LPA₂ receptor did not influence the seeding of lung

metastasis (Fig. 4C and D). On the contrary, compared to WT mice, LPA₅ KO mice showed substantially fewer metastases, with most of the mice having no detectable lung metastases (Fig. 4E and F). Although the lack of available antagonists for LPA₅ receptor impeded further validation of these studies, these data from KO mice nonetheless demonstrate that host LPA₅ receptors may potentially be involved in the homing of metastasizing cells. Analysis of LPA₁₋₅ receptor expression profiles in whole lung tissue isolated from the respective knockout mice revealed no changes in the expression of LPA₂, LPA₄ and LPA₅ across genotype. Only LPA₁ expression was moderately elevated in LPA₅ KO mice, whereas LPA₃ expression was increased in LPA₂ KO mice (Supplementary Fig. 2).

We also examined the effect of host LPA₁, LPA₂, and LPA₅ receptors on tumor growth. However, no differences in the subcutaneous growth of B16F10 cells were observed between WT and the respective LPA receptor KO mice (Fig. 5A-F).

Differential lung distribution of B16F10 cells at early time points in LPA₁- and LPA₅-KO mice

To gain insight into why lung metastases were reduced in LPA₁- and LPA₅-KO mice, we examined the distribution of B16F10 cells in mice at 24 h post-inoculation. To visualize cells at early time points, we transduced B16F10 cells with a GFP construct. The expression profiles of ATX and LPA receptors in GFP-B16F10 cells were unaltered, and the cells retained their ability to form lung metastasis when injected into mice (Supplementary Fig. 3). At 24 h post-injection, lungs were isolated, and GFP-B16F10 cells on the lung surface were visualized and quantified using an inverted fluorescent microscope. As shown in Fig. 6A and B, fewer GFP-B16F10 cells were seen on the lung surfaces of LPA₁- and LPA₅-KO mice, respectively. On the contrary, GFP-B16F10 cells appeared to be distributed to the same degree in both WT and LPA₂ KO mice (Fig. 6A). No fluorescent cells were detected in control mice injected with unlabeled B16F10 cells (Fig. 6B). We demonstrated that the differences seen in the distribution of GFP-B16F10 cells in LPA₁- and LPA₅-KO mice were significant (Fig. 6C and D).

Discussion

In the present study, we showed that tumor- and stromal-LPA receptors play different roles in the invasion and metastasis of malignant melanoma. Our finding that LPA inhibits B16F10 invasion via the LPA₅ receptor clearly supports recent data from Jongsma and colleagues, who demonstrated a similar anti-migratory effect of LPA₅ in these cells *in vitro* (30). However, the anti-invasive attribute of LPA₅ seen in these *in vitro* studies did not translate to *in vivo* observations. Even though knockdown of LPA₅ in B16F10 cells did relieve the inhibitory effect of LPA on invasion, the magnitude of effect we observed in these *in vitro* assays was moderate (as shown in Fig. 2B), which could possibly explain the lack of differences seen in the *in vivo* studies. Another possible explanation for the discrepancy between the *in vitro* and *in vivo* findings is the involvement of additional pro-invasive mechanism(s) *in vivo* that are independent of LPA, which may override the anti-invasive attribute of LPA₅ in B16F10 cells.

We also questioned the role of LPA₆ and LPA₂ on B16F10 invasion. Jongsma and colleagues ruled out the involvement of LPA₆ in invasion, as knockdown of the receptor affected neither the basal invasion rate nor the inhibitory actions of LPA on B16F10 invasion (30). On the contrary, our data with the siRNA-mediated knockdown of LPA₂ or the LPA₂ antagonist compound 35 showed that the basal invasion rate of B16F10 was clearly reduced by blocking LPA₂. Since these experiments were performed under serum-free conditions, one might question where the source of LPA is from and if ATX activity accounted for the basal invasion of B16F10 cells. We found that BMP22 dose-dependently reduced the rate of basal invasion in these cells (Supplementary Fig. 4). This suggests that B16F10 cells are capable of providing a source of substrate for ATX thereby generating its own pool of LPA for the activation of LPA₂. Indeed, early studies have shown that cancer cell lines such as human A2058 melanoma cells and MDA-MB-231 breast cancer cells released detectable amounts of LPC into the culture medium (3), which could arguably be the case for B16F10 cells. The role of LPA in the regulation of matrix metalloproteinase (MMP) is well documented. In particular, the ATX-LPA-LPA₁ signaling axis has been shown to induce MMP-9 expression in hepatocellular carcinoma (HCC) subsequently enhancing the invasive capacity of these cells (42). Similarly, studies by Do et al., reported a role for LPA in regulating MMP-2 activity in epithelial ovarian cancer (EOC) (43). Because MMPs play a vital role in invasion by degrading the extracellular matrix, it would be interesting to assess if LPA receptors differentially regulate MMP expression or activity in B16F10 cells.

Although we have unraveled a distinct role for host LPA₁, LPA₂, and LPA₅ receptors in supporting the seeding of lung metastasis by B16F10 cells, the real challenge ahead lies in deciphering the mechanism(s) of action. The experimental metastasis model we used herein studies the late stages of the metastatic cascade. Nonetheless, it is still a complex process in which tumor cells must survive the harsh conditions in the systemic circulation, evade the host immune system, arrest or adhere at a distal vessel wall, and extravasate into the surrounding tissue to establish secondary metastasis (31). Hence, there is a possibility that host LPA₁ and LPA₅ receptors could be involved in any of these steps. Nevertheless, our observation that LPA₁- and LPA₅-KO mice had diminished GFP-B16F10 residence in the lungs at an early time point (24 h) suggests that an impaired tumor-platelet interaction, tumor-endothelial cell interaction or adhesion may be involved.

We found that mesothelial cells isolated from LPA₁ KO mice were more resistant to invasion by MM1 hepatocarcinoma cells. Moreover, pharmacological inhibition of the LPA₁ receptor with Ki16425 significantly reduced the invasion of MM1 cells across the HUVEC monolayer. It is important to note that MM1 cells predominantly expressed LPA₂ and LPA₆ receptors, whereas the expression level of LPA₁ was low (32). Therefore, we postulate that Ki16425 most likely acts by antagonizing the LPA₁ receptor in HUVEC to reduce the invasion by MM1 cells; HUVEC express LPA₁ but not LPA₃ receptors, thus ruling out the effect of Ki16425 on LPA₃ receptors (44). In fact, earlier studies have shown that LPA is capable of causing endothelial barrier dysfunction and vascular permeability (45). A more recent study by Tager and colleagues described the involvement of LPA₁ receptors in increasing vascular leakage following tissue injury in a bleomycin mouse model of pulmonary fibrosis (46). Although neither of these studies was conducted in the context of

tumor-endothelial cell interaction, it certainly supports a positive role for the LPA-LPA₁ receptor axis in regulating endothelial barrier function. Nonetheless, more studies are needed to further validate the involvement of endothelial LPA₁ receptors in mediating tumor adhesion or invasion.

We determined that primary mouse lung microvascular endothelial cells (MLVEC) isolated from C57BL/6 mice predominantly express LPA₆, LPA₁, and LPA₄ receptors. Since these cells do not express LPA₅ receptors, we speculate that the diminished residence of GFP-B16F10 cells on the lung surface of LPA₅ KO mice at an early time point may not be caused by an impaired tumor-endothelial cell interaction. Moreover, we demonstrated that the rate of MM1 invasion across mesothelial cells isolated from LPA₅ KO mice was similar to that in WT mesothelium. Hence, what accounts for the reduced lung metastasis observed in LPA₅ KO mice remains unknown. We considered one possible mechanism of action based on recent work by Oda et al., who found that activation of LPA₅ receptors on cytotoxic CD8⁺ T cells inhibited T cell activation and proliferation. CD8⁺ T cells are a subset of immune cells that participate in tumor immunosurveillance; thus, inhibition of T cell activation may be advantageous for tumor cells in evading host immunity. Subsequently, the authors showed that the growth rate of established melanoma tumors in WT mice was reduced following the transfer of naïve LPA₅^{-/-} tumor-specific T cells (47). These findings suggest a potential role for LPA₅ in regulating host immunity toward cancer progression. Therefore, additional studies are needed to assess the response of the host immune system in LPA₅ KO mice toward metastasizing B16F10 cells. These studies should be focused on the early hours post tumor inoculation because by 24 h only few B16F10 cells can be detected in the lungs. Platelets interact with tumor cells and this interaction has been shown to increase LPA production and LPA-mediated bone metastasis formation by breast cancer cells (48). LPA₅ has been shown to mediate platelet activation (40) and the lack of this receptor subtype in platelets might also impact LPA production in the tumor microenvironment. This hypothesis should be tested in subsequent studies.

To understand how host LPA receptors may potentially be educated by tumor cells to form a permissive microenvironment or “premetastatic niche,” we reviewed the LPA receptor expression profiles in other stromal elements of the lung microenvironment. Tager and colleagues have extensively profiled LPA receptor expression in fibroblast and leukocyte subsets of murine lungs. LPA₁ was the predominant receptor expressed in lung fibroblasts, whereas alveolar macrophages predominantly expressed LPA₅, followed by LPA₄ and, to a lesser extent, LPA₂ receptors. LPA₂ and LPA₅ were the most abundantly expressed receptors in CD4⁺ and CD8⁺ T lymphocytes (46). The majority of these cells play vital roles in shaping the microenvironment for cancer progression and metastasis, as reviewed in (49). It would be of interest to study whether host LPA receptors in these stromal elements affect TME interaction. We have also determined the ATX and LPA receptor expression in primary rat alveolar type II epithelial cells and differentiated type I-like epithelial cells (derived from type II cells), which line the pulmonary alveoli. We found that these cells express high levels of LPA₂, P2Y₁₀, and ATX and moderate levels of LPA₆, LPA₃, and LPA₅ (Supplementary Fig. 5).

In summary, we show for the first time that LPA₁- and LPA₅-KO mice are protected from lung metastasis in this model. Although presently there is a lack of experimental evidence to elucidate the mechanism(s) involved in the homing of metastasizing tumor cells by host LPA receptors, future studies in which expression of selective LPA receptors can be regulated in a cell-type manner should help to clarify this issue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

ATX and LPA receptor profiling in (A) B16F10 and (B) mouse lung vascular endothelial cells (MLVEC) using quantitative real-time PCR. Time course of ATX activity measured in the conditioned media (CM) of (C) B16F10 and (D) MLVEC. Control represents media that were not exposed to cells. Data are expressed as mean $\pm$ SD of an experiment performed twice in quadruplicates (for quantitative real-time PCR) and three times in triplicates for the ATX activity assay.

Figure 2.

Inhibition of B16F10 cell invasion by LPA is mediated through LPA₅. (A) Validation of shRNA knockdown of LPA₅ in B16F10 using quantitative real-time PCR. (B) Invasion of LPA₅ knockdown-B16F10 across a Matrigel layer in the presence or absence of increasing concentrations of LPA. (C) Validation of siRNA knockdown of LPA₂ in B16F10 using quantitative real-time PCR. (D) Invasion of LPA₂ knockdown-B16F10 or non-targeting scrambled siRNA-B16F10 (control) across a Matrigel layer. (E) Invasion of B16F10 in the presence of the LPA₂ antagonist, compound 35. (F) Validation of LPA₁ overexpression in B16F10 using quantitative real-time PCR. (G) Invasion of B16F10 cells overexpressing LPA₁ across a Matrigel layer in the presence or absence of increasing concentrations of LPA. Data are representative of an experiment performed twice in quadruplicates and are expressed as mean ± SD. *Denotes *p* value < 0.05 using one-way ANOVA followed by a Bonferroni post-test. (H) Effect of LPA₅ knockdown-B16F10 cells on lung metastasis in C57BL/6 mice. *n* = 22 mice injected with scrambled shRNA B16F10 cells, and *n* = 21 mice injected with shLPA₅ B16F10 cells.

Figure 3.

Role of host LPA receptors in cell invasion and the seeding of metastasis. (A) MM1 rat hepatoma cell invasion across a mesothelial monolayer isolated from LPA₁-, LPA₂-, and LPA₅-KO mice, respectively. (B) Ki16425 inhibits LPA-induced MM1 cell invasion of HUVEC monolayer. Data are expressed as mean ± SD of an experiment performed three times in duplicates. *Denotes *p* value < 0.05 using one-way ANOVA followed by a Bonferroni post-test. (C) Effect of Ki16425, BMP22, and combination therapy on the lung metastasis of B16F10 cells in C57BL/6 mice. Vehicle treatment: *n* = 9 mice, Ki16425: *n* = 8

mice, BMP22: $n = 8$ mice; and combination treatment of Ki16425 and BMP22: $n = 10$ mice. P values were obtained using one-way ANOVA followed by Newman-Keuls multiple comparison test.

Figure 4.

Effect of host LPA receptor on the homing of B16F10 lung metastasis. (A) LPA₁ KO mice had a reduced number of lung nodules. $n = 11$ WT mice, and $n = 8$ LPA₁ KO mice. (B) H&E staining of lungs from WT (left panel) and LPA₁ KO mice (right panel). (C) No differences in the number of lung nodules were observed between WT and LPA₂ KO mice. $n = 12$ WT mice, and $n = 9$ LPA₂ KO mice. (D) H&E staining of lungs from WT (left panel) and LPA₂ KO mice (right panel). (E) LPA₅ KO mice had a reduced number of lung nodules. $n = 10$ WT mice, and $n = 12$ LPA₅ KO mice. (F) H&E staining of lungs from WT (left

panel) and LPA₅ KO mice (right panel). *P* values are relative to control WT mice. Scale bar = 500 microns.

Figure 5.

Effect of host LPA receptor on the subcutaneous growth of B16F10. No significant differences in subcutaneous tumor growth were observed between WT and LPA₁ KO (A & B), LPA₂ KO (C & D), or LPA₅ KO (E & F) mice.

Figure 6.

Distribution of GFP-B16F10 cells in mice at early time points. (A & B) Representative images of GFP-B16F10 cells on the lung surfaces of WT, LPA₁-, LPA₂-, and LPA₅-KO mice, respectively. Control = injection with unlabeled B16F10 cells. Scale bar = 200 microns (C). Distribution of GFP-B16F10 cells in LPA₁-, LPA₂-, and (D) LPA₅-KO mice at 24 h after injection via tail vein. $n = 11$ WT mice, $n = 8$ LPA₁ KO mice, $n = 8$ LPA₂ KO mice, and $n = 10$ LPA₅ KO mice. P values are relative to control WT mice.