Autotaxin and LPA₁ and LPA₅ Receptors 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 LPA₁ receptors but lack LPA₂. 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 pharmacologic inhibition of LPA₁ 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 with wild-type (WT) mice. LPA₁-KO 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-KO and LPA₅-KO animals was apparent 24 hours after injection. However, KO of LPA₁, LPA₅, or LPA₂ did not affect the subcutaneous growth of melanoma tumors.

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. Mol Cancer Res; 13(1); 174–85. ©2014 AACR.

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 biologic fluids (2, 3). Notably, plasma LPA levels in heterozygous knockout (KO) mice for the ATX gene (atx¹/²) were reduced by half compared with 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 the biologic activities of ATX. This is not surprising, as early studies have demonstrated that LPA has protumorigenic 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 GPCRs that were reported to be activated by LPA, among which LPA₁, LPA₂, and LPA₅ receptors have been extensively studied. LPA₁–₅ receptors belong to the same endothelial differentiation gene (edg) GPCR subfamily as the sphingosine-1-phosphate receptors...
Role of ATX and LPA Receptors in the Tumor Microenvironment

Materials and Methods

Materials

LPA (18:1) and lysophosphatidylcholine (LPC; 18:1) were purchased from Avanti Polar Lipids. ATX inhibitor BMP22 and specific LPA3 antagonist compound 35 were synthesized as described in refs. (32) and (34). K16425 was purchased from Cayman Chemical. Stock solutions (10 mmol/L) of BMP22, compound 35, and K16425 were prepared in DMSO for in vitro studies. A stock solution of LPA (1 mmol/L) was prepared as an equimolar complex with charcoal-stripped, fatty acid–free bovine serum albumin (BSA; Sigma-Aldrich) in PBS. Polyethylene glycol 400 (PEG 400) was purchased from Sigma-Aldrich. All cell culture media were purchased from Corning Cellgro, and cell culture reagents were from Life Technologies unless otherwise stated.

The fluorescent ATX substrate FS-3 was purchased from Echelon Biosciences.

Cell culture

B16F10 melanoma cells (gift from Dr. Gordon Mills, University of Texas MD Anderson Cancer Center, Houston, TX) were cultured in Eagle’s Minimum Essential Medium (MEM) supplemented with 5% (v/v) heat-inactivated HyClone FBS (Thermo Scientific), 2 mmol/L L-glutamine, 1 × MEM vitamin, 1 × MEM nonessential amino acid, 1 mmol/L sodium pyruvate, and 1 × antibiotic–antimycotic. The highly invasive MM1 rat hepatoma suspension cells (gift from Dr. Michiko Mukai, Osaka University, Osaka, Japan) were grown in DMEM supplemented with 10% (v/v) FBS, 2 mmol/L 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. and cultured in MCD-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 (RLMVEC) from C57BL/6 mice were purchased from Cell Biologics, Inc. and cultured in M1668 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 (ATCC) were used for culture of ATII cells. Freshly isolated cells were seeded to confluence at 3.5 × 10^5 cells per well in ATII culture medium (DMEM with 10% FBS, 4 mmol/L L-glutamine, 1% penicillin/streptomycin, and 0.25 μmol/L 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 6, cells were harvested and total RNA was isolated using the RNA isolation kit, RNeasy (Qiagen).

shRNA knockdown in B16F10 melanoma cells

Five clones of lentiviral shRNA LPA3 constructs (SHCLNG-XM_355812) were purchased from Sigma-Aldrich. Viral particles

(17). Consistent with the reported roles of ATX and LPA in cancer, it seems apparent that LPA receptors are also involved in tumorogenesis. 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 LPA2 and LPA3 receptors are implicated in breast cancer progression; the LPA3 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 among samples (10).

In contrast to LPA1, little is known about the roles of LPA2 (GPR23/P2Y9), LPA4 (GPR23/P2Y9), 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 LPA2 in tumorogenesis. Specifically, work by Harper and colleagues demonstrated that the LPA3 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 LPA2 was found to inhibit cell motility and invasion of mouse embryonic fibroblasts (29). It has recently been shown that the LPA3 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 current 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 LPA3 and almost completely reduced by the absence of LPA2, whereas LPA2 and LPA3 expressed in the tumor cell promote and inhibit invasion, respectively.

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were produced by cotransfection of 293FT cells with an shRNA-expressing plasmid (CCCGGCTTCAGATCCACGCTGTTTACGTTACTC-
GAGTAAAACGTGCATCCGACGTCGTGTTTG), or pLKO 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 cells 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.

siRNA knockdown in B16F10 melanoma cells
siRNA pools specific for LPA1 and nontargeting scrambled
siRNA were purchased from Dharmacon (GE Healthcare).
B16F10 cells were plated onto a 35-mm dish at a density of 2 ×
10^5 cells in growth medium and incubated overnight. The fol-
lowing day, the cells were transfected with 30 pmol of siRNA using
Lipofectamine RNAiMAX reagent (Life Technologies) according
to the manufacturer's protocol. Twenty-four 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 GFP, empty
vector, or LPA1-containing lentiviruses, respectively, in the pres-
ence 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 1
week.

RNA isolation and real-time quantitative PCR
Total RNA was extracted using TRIzol reagent (Life Technolo-
gies) followed by treatment with DNase I (Sigma-Aldrich) to
remove any trace of genomic DNA contamination. Single-strand-
ed cDNA was synthesized using the Thermoscript RT System (Life
Technologies). Quantitative real-time PCR was performed using
StepOnePlus instrument (Applied Biosystems) 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
seconds and 60°C for 60 seconds. Each sample was normalized to
the relative expression levels of GAPDH. Primer sequences are
listed in Supplementary Table S1.

Measurement of ATX activity
Conditioned media (CM) from B16F10, MLVEC, MM1, 
HIVEC, RLMVEC or isolated murine mesothelial cells were
prepared, respectively, by incubating 5 × 10^5 cells for 20 hours
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 300 000 Centrifugal Filter Units (Millipore).
ATX/lyso-PLD activity was measured by incubating 20 μL of
concentrated CM with 2 μmol/L of the fluorogenic substrate FS-
3 and 10 μmol/L of BSA. The change in fluorescence intensity
was monitored for 3.5 hours 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 × 10^6 
HUVECs (passages 4–6) were seeded into each well of a
12-well plate precoated with 0.2% gelatin (Sigma-Aldrich)
and incubated for 2 days to form a confluent monolayer.
Mesothelial cells harvested from three mice were initially plated in
6-well plates precoated with poly-l-lysine (Sigma-Aldrich)
and grown to confluence in DMEM supplemented with 10% 
(V/V) FBS in the presence of 2 mmol/L 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 pretreated with 2 μg/mL calcein AM (Invitrogen) for
2 hours, rinsed once, and seeded at a density of 5 × 10^3 cells per
well over the monolayers. Tumor cells were left to invade the
monolayers for 24 hours 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, noninvaded 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 TI-U inverted microscope using
phase-contrast and fluorescence illumination in a minimum of
five nonoverlapping fields at 100 × magnification. The fluores-
cent images were overlaid on top of the phase-contrast images
using Elements BR software (version 3.1.x), and the invading
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). The Matrigel coating of the
plates was rehydrated with PBS for 2 hours 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 top 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
hours at 37°C. After incubation, the medium in the top chamber
was removed and the insert was transferred into a new 24-well
plate containing 4 μg/mL of calcein AM in Hank balanced salt
solution. The plates were incubated for 1 hour at 37°C. The
fluorescence of invaded cells was then measured with a FLEXSta-
tion 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 at the University of Ten-
nessee (Knoxville, TX) and were consistent with the Guide for
the Care and Use of Laboratory Animals (39). B16F10 cells
(1 × 10^3) 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, $1 \times 10^6$ B16F10 cells transduced with either lentivirus containing scrambled shRNA or shRNA for LPA$_5$ were injected into the tail vein of 8- to 12-week-old female C57BL/6 mice (purchased from The Jackson Laboratory). In experiments involving treatment with BMP22 and/or Ki16425, $1 \times 10^5$ B16F10 cells were injected into the tail vein of 8- to 12-week-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), or BMP22 (0.1 mg/kg), or a combination of Ki16425 and BMP22 via intraperitoneal injection daily for 20 days.

**Histopathologic 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 hematoxylin and eosin (H&E).

**Lung extravasation assay**

In some experiments, $1 \times 10^6$ 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 24 hours after injection. Lungs were harvested and filled with PBS, and the GFP-expressing B16F10 cells on the lung surface were imaged using a Nikon Ti inverted fluorescence microscope (using a low-magnification 4× 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, $4 \times 10^5$ B16F10 cells were injected subcutaneously into the left flank of each mouse in a 100 μl volume. The cells were prepared by reconstituting with Dulbecco’s modified Eagle medium (DMEM) and cultured for 24 hours before injection. Lungs were harvested and filled with PBS, and the GFP-expressing B16F10 cells on the lung surface were imaged using a Nikon Ti inverted fluorescence microscope (using a low-magnification 4× 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.

**Statistical analysis**

All statistical analyses were performed with GraphPad Prism software (version 5.0) using the two-tailed unpaired Student t test or the one-way ANOVA, followed by either a Bonferroni posttest 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 MLVECs. We found that B16F10 cells showed robust expression of transcripts encoding ATX, LPA$_2$, LPA$_5$, and LPA$_6$ receptors (Fig. 1A). In contrast, MLVECs had very low expression of ATX and LPA$_2$ transcripts and predominantly expressed LPA$_6$, LPA$_1$, and LPA$_5$ (Fig. 1B). We observed a similar trend in our previous studies in which expression of ATX was higher in MM1 rat hepatocarcinoma cells compared with expression of stromal cells such as HUVMECs and isolated murine mesothelial cells (32). Because ATX is primarily a secreted enzyme that hydrolyzes LPC to LPA, we decided to measure ATX activity in concentrated CM of B16F10 cells and MLVECs. To do this, we utilized the synthetic fluorogenic substrate FS-3, which was cleaved by ATX to release the fluorescent moiety from the quencher (40). 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 MLVECs (Fig. 1C and D). We extended this assessment to include concentrated CM from MM1 hepatocarcinoma, HUVMECs, RLMVECs, and mesothelial cells. Except the MM1 cells, we were unable to detect ATX activity in the CM from any of these stromal cells (Supplementary Fig. S1). On the basis of these observations, we concluded that B16F10 cells may provide a rich source of ATX within the tumor microenvironment.

**LPA$_5$ 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 LPA$_5$ receptor, the predominant LPA receptor expressed in B16F10 cells. This system effectively reduced the levels of LPA$_5$ mRNA by 80% compared with the levels in B16F10 cells transduced with scrambled shRNA (Fig. 2A). We found that knockdown of LPA$_5$ in B16F10 cells moderately attenuated the inhibitory effect of LPA on invasion. This effect was observed only when concentrations higher than 30 nmol/L LPA were used (Fig. 2B). In addition, the basal invasion rate of B16F10 cells was unaffected by the knockdown of LPA$_5$.

We next examined the role of the LPA$_2$ receptor in B16F10 cell invasion. To do this, we utilized a siRNA targeting system to knockdown LPA$_2$ in B16F10 by 75% (Fig. 2C) and assessed the effect on invasion. We found that knockdown of LPA$_2$ significantly reduced the basal invasion rate of B16F10 compared with the scrambled siRNA control (Fig. 2D). To complement this finding, we used a potent and selective small-molecule antagonist for LPA$_2$, designated as compound 35 by Beck and colleagues (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 LPA$_2$ 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 LPA$_5$ mRNA (Fig. 1A), with an LPA$_2$ construct using the lentiviral system (Fig. 2F). We hypothesized that introducing the proinvasive LPA$_2$ receptor might affect the outcome of invasion in these cells. Indeed, we noted that the invasion rate of LPA$_2$-overexpressing B16F10 cells in response to LPA increased significantly compared with the rate of vector-transduced B16F10 cells (Fig. 2G). In fact, cell invasion was greatest at 10 nmol/L of LPA, and it decreased in magnitude at higher concentrations of LPA. We reasoned that this might be attributed to the preference of LPA$_2$ receptors for the acyl form of LPA.
Host LPA1 and LPA3 receptors affect the homing of metastasizing B16F10 cells in vivo

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 (42). This model measures the invasion of MM1 rat hepatocarcinoma cells across a mesothelial monolayer that commonly lines the serous cavities of the body. 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 LPA1/3 receptor antagonist Ki16425 to cocultures of MM1 cells and HUVEC monolayers, and quantified invasion after 24 hours. 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, $1 \times 10^5$ 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 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 with 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 LPA3 receptor did not influence the seeding of lung metastasis (Fig. 4C and D). On the contrary, compared with WT mice, LPA3 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 LPA3 receptor impeded further validation of these studies, these data from KO mice nonetheless demonstrate that host LPA3 receptors may potentially be involved in the homing of metastasizing cells. Analysis of LPA1 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 LPA4 expression was moderately elevated in LPA2 KO mice, whereas LPA4 expression was increased in LPA3 KO mice (Supplementary Fig. S2).

We also examined the effect of host LPA1, LPA2, and LPA3 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).

**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 B16F10 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 nontargeting scrambled siRNA-B16F10 (control) across a Matrigel layer. E, invasion of B16F10 cells overexpressing LPA1 across a Matrigel layer in the presence or absence of increasing concentrations of LPA. F, invasion of B16F10 cells overexpressing LPA3 across a Matrigel layer in the presence or absence of increasing concentrations of LPA. G, invasion of B16F10 cells overexpressing LPA4 across a Matrigel layer in the presence or absence of increasing concentrations of LPA. 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.

**Discussion**

In the current 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 LPA3 receptor clearly supports recent data from Jongsma and colleagues, who demonstrated a similar antimigratory effect of LPA3 in these cells in vitro (30). However, the anti-invasive attribute of LPA3 seen in these in vitro studies did not translate to in vivo observations. Even though knockdown...
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 proinvasive mechanism(s) in vivo that are independent of LPA, which may override the anti-invasive attribute of LPA in B16F10 cells.

We also questioned the role of LPA1 and LPA5 on B16F10 invasion. Jongsm and colleagues ruled out the involvement of LPA 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 LPA. As these experiments were performed under serum-free conditions, one might question where the source of LPA is from and whether 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. S4). 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 LPA receptors. Indeed, early studies have shown that cancer cells 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–LPA, signaling axis has been shown to induce MMP9 expression in hepatocellular carcinoma subsequently enhancing the invasive capacity of these cells (43). Similarly, studies by Do and colleagues reported a role for LPA in regulating MMP2 activity in epithelial ovarian cancer (44). Because MMPs play a vital role in invasion by degrading the extracellular matrix, it would be interesting to assess whether 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 hours) 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, pharmacologic 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 LPA5 receptors, whereas the expression level of LPA1 was low (32). Therefore, we postulate that Ki16425 most likely acts by antagonizing the LPA1 receptor in HUVECs to reduce the invasion by MM1 cells; HUVEC express LPA1, but not LPA5 receptors, thus ruling out
In fact, earlier studies have shown that LPA is capable of causing endothelial barrier dysfunction and vascular permeability (46). 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 (47). Although neither of these studies was conducted in the context of tumor–endothelial cell interaction, it certainly supports a positive role for the LPA1–LPA3 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.

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) and LPA1-KO mice (right). 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) and LPA2-KO mice (right). 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) and LPA5-KO mice (right). P values are relative to control WT mice. Scale bar = 500 μm.
We determined that MLVECs isolated from C57BL/6 mice predominantly express LPA_6, LPA_1, and LPA_4 receptors. As these cells do not express LPA_5 receptors, we speculate that the diminished residence of GFP-B16F10 cells on the lung surface of LPA_5-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 LPA_5-KO mice was similar to that in WT mesothelium. Hence, what accounts for the reduced lung metastasis observed in LPA_5 KO mice remains unknown. We considered one possible mechanism of action based on recent work by Oda and colleagues, who found that activation of LPA_5 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 naive LPA_5−/− tumor-specific T cells (48). These findings suggest a potential role for LPA_5 in regulating host immunity toward cancer progression. Therefore, additional studies are needed to assess the response of the host immune system in LPA_5-KO mice toward metastasizing B16F10 cells. These studies should be focused on the early hours after tumor inoculation because by 24 hours only few B16F10 cells can be detected in the lungs.

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 LPA_1-KO (A and B), LPA_2-KO (C and D), or LPA_5-KO (E and F) mice.
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 (49). LPA5 has been shown to mediate platelet activation (41) 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 LPA3, 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 (47). The majority of these cells play vital roles in shaping the microenvironment for cancer progression and metastasis, as reviewed in ref. (50). 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 LPA3, P2Y10, and ATX and moderate levels of LPA6, LPA4, and LPA5 (Supplementary Fig. S5).

Figure 6.
Distribution of GFP-B16F10 cells in mice at early time points. A and B, representative images of GFP-B16F10 cells on the lung surfaces of WT, LPA1-, LPA2-, and LPA5-KO mice, respectively. Control represents lungs of mice injected with unlabeled B16F10 cells. Scale bar = 200 μm. C and D, distribution of GFP-B16F10 cells in LPA1-, LPA2-, and LPA5-KO mice at 24 hours 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.
In summary, we show for the first time that LPA1- and LPA3-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.

Disclosure of Potential Conflicts of Interest

G.J. Tigyi is chief scientist, has ownership interest (including patents), and consultative/advisory board member for Bellox Inc. No potential conflicts of interest were disclosed by the other authors.

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Autotaxin and LPA$_1$ and LPA$_5$ Receptors Exert Disparate Functions in Tumor Cells versus the Host Tissue Microenvironment in Melanoma Invasion and Metastasis

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