

RESEARCH HIGHLIGHT

Uncovering unique roles of LPA receptors in the tumor microenvironment

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The role of the lysophospholipase D autotoxin (ATX) and lysophosphatidic acid (LPA) in cancer is emerging and represents two key players in regulating cancer progression. In this brief review, we will discuss some of our recent findings, which highlight a central role that LPA and its receptor plays in orchestrating melanoma-stroma interactions in the establishment of lung metastases. In particular, we evaluated not only the function of LPA receptors on tumor cells but also their role on host tissues and how they can influence melanoma growth and metastasis. Using the syngeneic B16F10 murine melanoma model, we made three key observations. First, our *in vitro* findings demonstrate that LPA receptors, specifically LPA₂ and LPA₅ expressed in B16F10 cells appear to have opposing roles in cell invasion; the former seems to be responsible for the high basal invasion rate of B16F10 cells while the latter is anti-invasive upon exogenous LPA stimulation. Second, we observed a profound reduction in the incidence of pulmonary melanoma metastasis in LPA₁- and LPA₅-knockout (KO) mice, respectively, when compared to wild-type (WT) mice. Third, no differences in terms of subcutaneous tumor growth between LPA₁KO, LPA₅KO and WT mice were observed. These findings suggest that LPA receptors exert different functions in melanoma cells versus host tissues in terms of invasion and metastasis.

Keywords: Lysophosphatidic acid receptor; autotoxin; melanoma; tumor microenvironment; invasion; metastasis

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LPA and cancer

ATX, also known as ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (ENPP2) is the primary enzyme that is responsible for the production of LPA by cleaving the headgroups of lysophospholipids, primarily lysophosphatidylcholine (LPC). The physiological and pathological effects of LPA are mediated through activation of a series of G protein-coupled receptors (GPCR). At least six of these receptors termed LPA₁ to LPA₆ have been

characterized whereas little is known about the putative LPA GPCRs (GPR87, P2Y10 and GPR35) [1, 2].

There are many reports of ATX-LPA receptor signaling axis being implicated in various cancers such as breast [3], ovarian [4], prostate [5], hepatocellular carcinoma [6], glioblastoma multiforme [7] and melanoma [8]. In most cases, overexpression of ATX and/or LPA receptors in these cancers correlates with the degree of malignancy i.e. enhanced invasiveness, chemoresistance and metastatic

potential of tumors^[9, 10].

Why melanoma?

We and others have made several key observations that corroborate a central role for ATX-LPA receptor signaling axis in melanoma invasion and metastasis. First, studies by Stracke *et al.* identified ATX in the conditioned media of human A2058 melanoma cells, which potently stimulate cancer cell motility^[11]. Subsequent studies demonstrated that it is LPA, the product of ATX that is responsible for mediating melanoma cell migration and proliferation^[12]. Moreover, increased ATX expression was detected in metastatic melanoma specimens compared to basal and squamous cell carcinoma, and normal skin^[8]. Many efforts have since been made in developing ATX inhibitors and an excellent review on the chemical evolution of these inhibitors can be found in^[13].

Differential roles of LPA receptors in melanoma invasion

The majority of our work is based on a syngeneic model of a highly metastatic B16F10 murine melanoma. When injected intravenously into mice, these highly aggressive B16F10 cells readily metastasized to the lungs. We found that the expression of ATX was strikingly high in these cells and the enzymatic activity of ATX could be readily detected in the conditioned media. Moreover, we demonstrated that the metastatic capacity of B16F10 cells *in vivo* is, in part, dependent on ATX. In particular, treatment with an ATX inhibitor, BMP22 significantly reduced pulmonary metastasis in mice^[14]. These findings prompted us to examine if the LPA receptor signaling axis contributes to the invasive behavior of B16F10 cells.

We found that B16F10 cells predominantly expressed LPA₅, LPA₂ and LPA₆ receptor transcripts. We evaluated the influence of these receptors on cell invasion using a matrigel-coated Boyden chamber assay system. In serum-free conditions, B16F10 cells exhibit a high basal invasion rate across the matrigel layer. However, when exogenous LPA was added as a chemoattractant, basal cell invasion was greatly attenuated. This observation was somewhat perplexing since one would expect exogenous LPA to enhance cell invasion. To examine which LPA receptors was responsible for the inhibitory effect of LPA on B16F10 invasion, we knocked down LPA₅ or LPA₂, using shRNA- and siRNA-directed methods. Interestingly, we noticed that the inhibitory effect of LPA on B16F10 invasion *in vitro* was relieved upon knockdown of LPA₅. An independent study conducted by Jongsma and colleagues also demonstrated a similar anti-migratory effect of LPA₅ in these cells. In addition, the authors showed that alkyl-LPA, which is the

preferred ligand for LPA₅^[15] was 10 fold more potent than acyl-LPA in inhibiting the migration of B16F10 cells^[16]. These findings suggest that activation of the LPA₅ receptor by high concentrations of acyl-LPA inhibits B16F10 cell invasion.

On the contrary, knockdown of LPA₂ but not LPA₅ was sufficient to cause a decrease in basal cell invasion. Similar results were obtained using a LPA₂ antagonist termed compound 35 developed by Beck and colleagues^[17]. Thus, LPA₂ appears to mediate the high basal invasion rate of B16F10 cells. Since no exogenous chemoattractant was used in these experiments, one might question what the source of LPA is. Based on evidence that B16F10 cells express and secrete high amounts of ATX, we postulate that these cells might be capable of generating their own pool of LPA for the activation of LPA₂. Indeed, we found that treatment of B16F10 cells with the ATX inhibitor BMP22 dose-dependently reduced basal cell invasion. Although we have not measured the levels of LPC in the culture media of B16F10 cells, studies by Umezu-Goto *et al.*, detected considerable amounts of LPC being released into the culture medium of human A2058 melanoma and MDA-MB 231 breast cancer cells^[12]. The release of LPC in the culture medium could in turn serve as a substrate for ATX.

B16F10 cells also express the LPA₆ receptor. Jongsma and colleagues have evaluated the contributory role of this receptor in B16F10 cell invasion. These authors found that knockdown of LPA₆ expression in B16F10 cells did not affect the basal invasion rate or the inhibitory actions of exogenous LPA on cell invasion^[16]. Taken together, these results appear to suggest that the differential expression and activation of LPA receptors by acyl- and/or alkyl-LPA might determine the outcome of invasion in B16F10 cells. We further tested this hypothesis by transducing B16F10 cells, which normally do not express LPA₁ receptor, with a LPA₁ construct. By overexpressing LPA₁ receptor in these cells, we were able to shift the LPA-induced invasive response from one that is chemorepulsive (upon activation of LPA₅) to one that is pro-invasive. These findings on the pro-invasive role of LPA₁ in melanoma cells corroborate the findings of LeBlanc *et al.* in breast cancer cells^[18].

LPA and the tumor microenvironment

There is a growing appreciation that the host microenvironment of a tumor plays a fundamental role in cancer progression. The notion is that tumor cells are capable of re-educating their host environment to form one that is “permissive”, allowing them to evade host immunity, grow and metastasize. Various stromal elements such as endothelial cells, fibroblasts, infiltrating immune cells, and

extracellular matrix make up the tumor microenvironment [19]. We reviewed the LPA receptor expression profile particularly in stromal cells of the lung microenvironment and found LPA receptors to be ubiquitously expressed. For example, we determined that primary mouse lung microvascular endothelial cells isolated from C57BL/6 mice predominantly expressed LPA₆, LPA₁ and LPA₄ receptors. Moreover, primary rat alveolar type II- and differentiated type I-epithelial cells, which line the pulmonary alveoli, expressed high levels of LPA₂, and moderate levels of LPA₆, LPA₃ and LPA₅. Studies by Tager *et al.* showed that lung fibroblasts predominantly expressed LPA₁, whereas alveolar macrophages expressed LPA₅, LPA₄ and LPA₂. These authors also reported abundant expression of LPA₂ and LPA₅ in CD4⁺ and CD8⁺ T lymphocytes [20]. Based on these findings, we were interested to study if stromal LPA receptors contribute to melanoma progression and metastasis.

The availability of LPA₁, LPA₂ and LPA₅ receptor knockout mice allowed us to address this aim. In our initial experiments, we isolated mesothelial cells from the respective LPA receptor KO mice and performed a transcellular invasion assay [21, 22]. In this model, the isolated mesothelial cells were cultured into a monolayer and served as a cellular barrier towards the invading tumor cells. We chose MM1 rat hepatocarcinoma cells over B16F10 as a source of tumor cells for the transcellular invasion assay with good reason. MM1 are suspension cells; however, they exhibit a distinct flattened morphology when they invade across the endothelial monolayer and remain associated to it. This allows for easy distinction between non-invaded (rounded cells) and invaded cells (flattened morphology). Moreover, the non-invaded floating cells can be removed from the culture medium by gently rinsing the mesothelial monolayer with PBS. Interestingly, we found that mesothelial cell monolayers isolated from LPA₁KO mice were more resistant to MM1 cell invasion. On the contrary, no differences in the invasion of MM1 cells were observed across mesothelial cells isolated from WT, LPA₂- or LPA₅-KO mice.

We were able to recapitulate this observation in HUVEC cells using the LPA₁/LPA₃ receptor antagonist, Ki16425. In particular, Ki16425 effectively reduced MM1 tumor cell invasion across the HUVEC monolayer. Since MM1 cells predominantly express LPA₂ and LPA₆ receptors, we presume that Ki16425 is most likely acting on the LPA₁ receptors that are expressed in HUVEC cells. Moreover, HUVEC cells lack LPA₃ expression, thus suggesting the effect of Ki16425 is mainly via LPA₁. These *in vitro* results seemingly indicate that the lack of LPA₁ or the inhibition of this receptor on stromal cells offers some level of protection against tumor cell invasion. To see if these *in vitro*

observations can be translated *in vivo*, we injected B16F10 cells into the tail vein of WT and LPA₁KO mice, respectively, and quantified lung metastases at day 21. We found that LPA₁KO mice had significantly fewer B16F10 lung metastases when compared to WT mice. Once again, these results can be recapitulated using Ki16425. Treatment with Ki16425 effectively reduced the number of lung nodules in WT mice inoculated with B16F10 cells. Because B16F10 cells lack LPA₁ and LPA₃ expression, we postulate that the effect of Ki16425 in reducing lung metastasis is mostly due to inhibition of LPA₁ receptors in host cells.

When we extended these *in vivo* studies to include the LPA₂- and LPA₅-KO mice, we found that the extent of B16F10 lung metastasis was the same between LPA₂KO mice and their WT counterparts. Intriguingly, lung metastasis was almost completely abolished in the LPA₅KO mice. This was the first demonstration that the homing of B16F10 melanoma cells to the lungs and seeding of metastases is substantially reduced by the absence of host LPA₁ and almost completely reduced by the absence of LPA₅. We also questioned whether host LPA receptor affects the subcutaneous growth of B16F10 *in vivo*. We found that neither tumor volume nor mass showed significant differences in the respective LPA KO and WT mice, suggesting that deletion of host LPA₁, LPA₂ or LPA₅ have limited effect on local tumor growth.

What's next?

Although our study demonstrates that host LPA receptors, specifically LPA₁ and LPA₅ are critical in supporting the establishment of lung metastasis, several key questions remain to be addressed: which step of the metastatic cascade is affected by host LPA₁ or LPA₅ receptors? Which stromal elements are involved in the process? In an attempt to address some of these questions, we performed preliminary experiments to examine the early distribution of fluorescently labeled GFP-tagged B16F10 cells in mice at 24 hour post-inoculation. Using fluorescence microscopy to image the GFP-B16F10 cells on the isolated lung surface, we found that fewer GFP-B16F10 cells were seen on the lung surfaces of LPA₁- and LPA₅-KO mice, when compared to WT mice. On the contrary, no differences were observed in the degree of GFP-B16F10 distribution in the lung surfaces of WT and LPA₂KO mice. These data seemingly suggest that deletion of host LPA₁ or LPA₅ probably affects the metastatic cascade at an early time point. For example, an impaired tumor-platelet interaction, tumor-macrophage interaction, or tumor-endothelial cell interaction or adhesion might be involved. On the basis of this, together with earlier results demonstrating that mesothelial cells isolated from LPA₁KO mice were more resistant to tumor cell invasion and

that primary mouse lung microvascular endothelial cells predominantly express LPA₁, we postulate that the deletion of host LPA₁ receptor most likely affects the tumor-endothelial interaction.

In support of this, several studies have demonstrated a critical role for LPA in regulating endothelial barrier function. In particular, studies by Amerongen *et al.* showed that LPA was capable of disrupting the endothelial barrier and as a result, increasing vascular permeability [23]. In addition, Tager and colleagues found that the LPA₁ receptor was responsible for the increase in vascular leakage following tissue injury in a mouse model of pulmonary fibrosis [20]. These findings certainly warrant for more studies to be conducted to validate the involvement of endothelial LPA₁ receptors, especially in the context of tumor-endothelial interaction, adhesion and invasion.

On the other hand, what accounts for the reduced GFP-B16F10 distribution on the lung surfaces of LPA₅KO mice at early time points and the finding that lung metastasis was almost completely abolished at day 21 remains unknown. Nonetheless, we postulate that host LPA₅ might affect the homing of metastasizing B16F10 cells in a different manner from that of host LPA₁ based on two key findings. First, primary mouse lung microvascular endothelial cells lack LPA₅ expression. Second, the rate of MM1 cell invasion across the isolated mesothelial monolayer was the same between LPA₅KO and WT mice. A recent study by Oda *et al.* may provide some indication as to what might be happening in LPA₅KO mice. These authors showed that cytotoxic CD8⁺ T cells, which are the subset of immune cells responsible for tumor immunosurveillance, predominantly express LPA₅ receptor. Activation of LPA₅ on CD8⁺ T cells inhibited T cell activation and proliferation, and as a result, allows for tumor cells to evade the host immunity. Moreover, the authors elegantly demonstrated that transfer of naïve LPA₅^{-/-} tumor-specific T cells successfully reduced the growth of established melanoma tumors in WT mice [24]. Taken together, these findings suggest a new and important role for LPA₅ in the regulation of host immunity towards cancer cells. Apart from cytotoxic CD8⁺ T cells, interaction of tumor cells with platelets is also an important step in the metastatic cascade. In particular, studies by Boucharaba *et al.*, showed that interaction of MDA-MB 231 breast cancer cells with platelets caused an increased in LPA production, which subsequently drove the formation of osteolytic bone metastasis in mice [25]. In view of this, a role for LPA₅ in mediating platelet activation has been reported [15], thus it would be interesting to examine the effect of LPA₅ knockdown in platelets in terms of LPA production and tumor-platelet interaction.

The findings from our study points to a new direction in which the understanding of stromal LPA receptors in the tumor microenvironment is equally as important as studying LPA receptors on the tumor cells. The major focus from now on would be to elucidate the mechanism(s) involved in the seeding of metastasizing tumor cells by host LPA receptors and to pinpoint the stromal elements that are implicated in this process.

Conflict of interest

The authors declare no competing financial interests.

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References

1. Choi JW, Herr DR, Noguchi K, Yung YC, Lee C-W, Mutoh T, *et al.* LPA Receptors: Subtypes and Biological Actions. *Annu Rev Pharmacol Toxicol* 2010;50:157-186.
2. Tigyi G. Aiming drug discovery at lysophosphatidic acid targets. *Br J Pharmacol* 2010;161:241-270.
3. Euer N, Schwirzke M, Evtimova V, Burtscher H, Jarsch M, Tarin D, *et al.* Identification of genes associated with metastasis of mammary carcinoma in metastatic versus non-metastatic cell lines. *Anticancer Res* 2002;22:733-740.
4. So J, Wang FQ, Navari J, Schreher Jand Fishman DA. LPA-induced epithelial ovarian cancer (EOC) in vitro invasion and migration are mediated by VEGF receptor-2 (VEGF-R2). *Gynecol Oncol* 2005;97:870-878.
5. Nouh MA, Wu XX, Okazoe H, Tsunemori H, Haba R, Abou-Zeid AM, *et al.* Expression of autotaxin and acylglycerol kinase in prostate cancer: association with cancer development and progression. *Cancer Sci* 2009;100:1631-1638.
6. Wu JM, Xu Y, Skill NJ, Sheng H, Zhao Z, Yu M, *et al.* Autotaxin expression and its connection with the TNF-alpha-NF-kappaB axis in human hepatocellular carcinoma. *Mol Cancer* 2010;9:71.
7. Kishi Y, Okudaira S, Tanaka M, Hama K, Shida D, Kitayama J, *et al.* Autotaxin is overexpressed in glioblastoma multiforme and contributes to cell motility of glioblastoma by converting lysophosphatidylcholine to lysophosphatidic acid. *J Biol Chem* 2006;281:17492-17500.
8. Altman MK, Gopal V, Jia W, Yu S, Hall H, Mills GB, *et al.* Targeting melanoma growth and viability reveals dualistic functionality of the phosphonothionate analogue of carba cyclic phosphatidic acid. *Mol Cancer* 2010;9:140.
9. Houben AJSand Moolenaar WH. Autotaxin and LPA receptor signaling in cancer. *Cancer Metastasis Rev* 2011;30:557-565.
10. Willier S, Butt Eand Grunewald TG. Lysophosphatidic acid (LPA) signalling in cell migration and cancer invasion: a focussed review and analysis of LPA receptor gene expression on the basis of more than 1700 cancer microarrays. *Biol Cell* 2013;105:317-333.

11. Stracke ML, Krutzsch HC, Unsworth EJ, Arestad A, Cioce V, Schiffmann E, *et al.* Identification, purification, and partial sequence analysis of autotaxin, a novel motility-stimulating protein. *J Biol Chem* 1992;267:2524-2529.
12. Umezu-Goto M, Kishi Y, Taira A, Hama K, Dohmae N, Takio K, *et al.* Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J Cell Biol* 2002;158:227-233.
13. Albers HMGand Ovaa H. Chemical Evolution of Autotaxin Inhibitors. *Chem Rev* 2012;112:2593-2603.
14. Gupte R, Patil R, Liu J, Wang Y, Lee SC, Fujiwara Y, *et al.* Benzyl and naphthalene methylphosphonic acid inhibitors of autotaxin with anti-invasive and anti-metastatic activity. *ChemMedChem* 2011;6:922-935.
15. Williams JR, Khandoga AL, Goyal P, Fells JI, Perygin DH, Siess W, *et al.* Unique Ligand Selectivity of the GPR92/LPA5 Lysophosphatidate Receptor Indicates Role in Human Platelet Activation. *J Biol Chem* 2009;284:17304-17319.
16. Jongsma M, Matas-Rico E, Rzadkowski A, Jalink Kand Moolenaar WH. LPA is a chemorepellent for B16 melanoma cells: action through the cAMP-elevating LPA5 receptor. *PLoS One* 2011;6:e29260.
17. Beck HP, Kohn T, Rubenstein S, Hedberg C, Schwandner R, Hasslinger K, *et al.* Discovery of potent LPA2 (EDG4) antagonists as potential anticancer agents. *Bioorg Med Chem Lett* 2008;18:1037-1041.
18. Leblanc R, Lee SC, David M, Bordet JC, Norman DD, Patil R, *et al.* Interaction of platelet-derived autotaxin with tumor integrin alphaVbeta3 controls metastasis of breast cancer cells to bone. *Blood* 2014; 124:3141-3150.
19. Quail DFand Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* 2013;19:1423-1437.
20. Tager AM, LaCamera P, Shea BS, Campanella GS, Selman M, Zhao Z, *et al.* The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat Med* 2007;14:45-54.
21. Akedo H, Shinkai K, Mukai M, Mori Y, Tateishi R, Tanaka K, *et al.* Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: a model for tumor invasion. *Cancer Res* 1986;46:2416-2422.
22. Mukai M, Nakamura H, Tatsuta M, Iwasaki T, Togawa A, Imamura F, *et al.* Hepatoma cell migration through a mesothelial cell monolayer is inhibited by cyclic AMP-elevating agents via a Rho-dependent pathway. *FEBS Lett* 2000;484:69-73.
23. Amerongen GPvN, Vermeer MAand van Hinsbergh VWM. Role of RhoA and Rho Kinase in Lysophosphatidic Acid-Induced Endothelial Barrier Dysfunction. *Atertio Thromb Vasc Biol* 2000;20:e127-e133.
24. Oda SK, Strauch P, Fujiwara Y, Al-Shami A, Oravec T, Tigyi G, *et al.* Lysophosphatidic Acid Inhibits CD8 T-cell Activation and Control of Tumor Progression. *Cancer Immunology Research* 2013;1:245-255.
25. Boucharaba A, Serre C-M, Grès S, Saulnier-Blache JS, Bordet J-C, Guglielmi J, *et al.* Platelet-derived lysophosphatidic acid supports the progression of osteolytic bone metastases in breast cancer. *J Clin Invest* 2004;114:1714-1725.