LPA-mediated autocrine signaling loop as a potential immune escape mechanism in human melanoma

PhD thesis

Enikő Major, PharmD

Doctoral School of Theoretical and Translational Medicine

Semmelweis University

Supervisor:	Zoltán Benyó, MD, DSc
Consultant:	Gábor J. Tigyi, MD, DSc
Official reviewers:	Eleonóra Nardainé Imrédi, MD, PhD József Tóvári, PhD
Head of the Complex Examination Committee:	György Losonczy, MD, DSc
Members of the Complex Examination Committee:	Andrea Székely, MD, PhD Ákos Jobbágy, DSc

Budapest
2024

1. Introduction

The incidence of melanoma is continuously increasing in the Western World and severely impacts patients' life quality and expectancy. A better understanding of the molecular mechanisms of tumor progression and anti-tumor immune response is essential to improve existing therapies and develop new ones for patients with melanoma and other cancers. Lysophosphatidic acid (LPA) is a bioactive lipid mediator produced in large amounts by melanoma and is abundantly present in the tumor microenvironment. LPA regulates a wide range of physiological and pathological cellular functions in almost every cell type via its six G-protein coupled receptors known as LPAR1-6, which can activate several intracellular signaling pathways. In the plasma, LPA is mainly generated from circulating pools of lysophospholipids, primarily from lysophosphatidylcholine, by autotaxin (ATX, encoded by ENPP2), a lysophospholipase D enzyme. ATX and LPAR expression is upregulated in several cancer types and mediates various aspects of carcinogenesis.

Death receptor 6 (DR6, encoded by TNFRSF21) is a type I transmembrane receptor that belongs to the tumor necrosis factor superfamily. Its mRNA is expressed in various organs, including the brain, heart, pancreas, and placenta. Since elevated expression of DR6 has been found to play a pivotal role in numerous human diseases, including Alzheimer's disease, inflammation, and autoimmune disease, it is considered a potential therapeutic target. Similarly, abundant transcript levels of DR6 were observed in several human cancers, indicating a role in tumor biology. DR6 has also been implicated in pro-apoptotic signaling. Interestingly, Dong et al. demonstrated that LPA is able to induce apoptosis via the upregulation of DR6 in HeLa cells, although other investigators did not confirm this effect under the same conditions.

Interleukin 10 (IL-10) is primarily recognized as an anti-inflammatory cytokine secreted by immune cells. It was later discovered that non-immune cell types, including various tumors, such as breast, colon carcinoma, and melanoma, can also produce IL-10. Although the role

of IL-10 in tumor biology is controversial, elevated serum IL-10 levels are reportedly associated with a poor prognosis in melanoma.
Tumors can evade immunosurveillance through several mechanisms, including altering the expression of immunologically relevant cell surface molecules and/or creating an immunosuppressive microenvironment by accumulating metabolites, cytokines, and chemokines. Although immune checkpoint inhibitors (ICIs) have improved melanoma survival rates, approximately 50% of patients with metastatic melanoma do not respond well to ICIs. Recently, a critical connection was discovered between melanomas with low expression levels of the human leukocyte antigen DR (HLA-DR), an MHC class II antigen, and reduced response rate to immunotherapies.

2. Objectives

We aimed to investigate the relation between LPA-LPAR signaling and IL-10-HLA-DR expression, which represents a previously unexplored topic in melanoma research. As elevated IL-10 levels are frequently associated with poor prognosis in different cancer types, we aimed to examine the potential role of LPA-induced IL-10 release and clarify the underlying signaling mechanisms in human melanoma. Specifically, we addressed the following questions:

- To investigate the effect of LPA on human DR6 promoter activity, transcription, and protein levels.
- To evaluate the LPA-related signaling in DR6 upregulation and assess the transcription factor mediating the increased transcription.
- To evaluate the effect of LPA on IL-10 transcription and release in human melanoma cells.
- To analyze the LPA-NF-κB-DR6-IL-10 axis in melanoma tumor samples to confirm the *in vitro* findings of the LPA-mediated signaling cascade.
- To investigate HLA-DR expression in melanoma cells to prove the importance of LPA-related IL-10 release.
- To analyze the correlation between LPAR1 expression and the efficiency of anti-PD-1 therapy in melanoma tumor samples in order to confirm the effect of LPA on HLA-DR expression.

3. Methods

3.1 Reagents

LPA 18:1 was purchased from Avanti Polar Lipids Inc (Alabaster, AL, USA) and dissolved in 1 mM fatty acid-free bovine serum albumin (BSA, Merck KGaA; Darmstadt, Germany). AM095 (118), Ki16425 (119), and pertussis toxin were obtained from Cayman Chemicals (Ann Arbor, MI, USA).

3.2 Cell culture

Human embryonic kidney HEK293T (RRID: CVCL_0063), as well as A2058 (RRID: CVCL_1059) and A375 (RRID: CVCL_0132) human melanoma cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin and were cultured in a humidified incubator at 37°C and 5% CO2. Each cell line underwent regular mycoplasma screening, and all experiments were performed using mycoplasma-free cells.

3.3 LPA treatment

In all experiments, cells were serum-starved for 1 hour prior to the administration of LPA. For inhibition of LPAR, the cells were pretreated with 10 μM AM095 or Ki16425, targeting LPAR1 or LPAR1/3 respectively, for 30 minutes prior to treatment with LPA. To investigate Gi protein coupling, the cells were preincubated with 100 ng/mL pertussis toxin (PTX) for 16 hours prior to LPA administration. Anti-IL-10 neutralizing antibody (JES3-9D7) or IgG1 isotype control (Thermo Fisher Scientific) was applied at 3.5 μg/mL, 1 hour prior to LPA treatment.

3.4 Luciferase Assay

Genomic DNA was isolated from human keratinocytes using DNeasy Blood&Tissue kit (Qiagen) and used as a template to

amplify the predicted hDR6 promoter using a forward and a reverse primer with the sequences:
5'-TCCATCGAGCTCTTGGGGGAAGGGTGATTAAA-3' and
5'-AAAACTCGAGTTCTGCCCAGCGCCGCATCCACC-3',
respectively. The amplicon was cloned between the SacI and XhoI restriction sites of the pGL4.10 (Promega, Madison, WI, USA) luciferase expression vector. All constructs were confirmed by sequencing.
HEK293T cells were cultured in 96-well plates. Twenty-four hours after seeding, the cells were co-transfected with the hDR6p-pGL4.10-luc luciferase expression vector and pRL Renilla luciferase control reporter driven by the SV40 promoter (Promega). Plasmid transfection was performed using Lipofectamine3000 (Invitrogen, Karlsruhe, Germany) in OptiMEM (Invitrogen) without supplements, according to the manufacturer's protocol. After 24 hours, cells were kept in a serum-free medium for one hour and treated with 10 μM LPA for the indicated times. Luciferase activities were measured using the Dual-Glo Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The relative luciferase activity was calculated by normalizing it to Renilla luciferase activity.

3.5 Gene knockdown

Small interfering RNA (siRNA) targeting DR6 (Catalog ID: L-004450-00-0005), IL10 (Catalog ID: L-005066-00-0005) or NF-κB1 (Catalog ID: L-003520-00-0005) mRNA (ON-TARGETplus SMARTpool), and non-targeting control (siNC) were purchased from Dharmacon (Lafayette, CO, USA). siRNAs were applied at the time of cell plating in 25 nM final concentration using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Treatments and measurements were carried out 24 h after transfection.

3.6 Quantitative RT-PCR

RNA was isolated from cells using the NucleoSpin RNA Plus XS kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). RNA concentration and quality were assessed with Nanodrop (Thermo Fisher Scientific). Up to 1 μg of total RNA was converted to cDNA

using the RevertAid First Standard cDNA synthesis kit (Thermo Scientific). RNA expression relative to GAPDH was assessed by quantitative real-time PCR using cDNA corresponding to 40 ng RNA. Reactions were performed with 250 nM of each forward and reverse primers in a final volume of 10 µl of 2XSsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA, USA). Amplification was performed after one initial denaturation step for 3 min at 98 ºC for 40 cycles at 95 ºC/10 s and 60 ºC/20 s with a CFX Connect™ Real-Time PCR Detection System (BioRad). The fold change of DR6 or IL-10 gene expression normalized to the housekeeping gene (GAPDH) in LPA-treated versus untreated control cells was defined as 2-ΔΔCT. The LPAR1-6 gene expressions were defined as 2-ΔCT. The primer sets used were as follows (Merck KGaA; Darmstadt, Germany):

Table 1. Primer sequences used in the quantitative RT-PCR analyses.

Genes	**Fwd 5'-3'**	**Rev 5'-3'**
GAPDH	TCGGAGTCAACGGATTTG	CAACAATATCCACTTTACCAGAG
DR6	GGCATGAACTCAACAGAATC	GTTGACTACCTGAAGGTTTG
IL-10	GCCTTTAATAAGCTCCAAGAG	ATCTTCATTGTCATGTAGGC
LPAR1	TACAGCATCAGGTACACAG	ATTACAGGGATGGAAGTAGAG
LPAR2	ACTGTTGTCATCATCCTGG	ACTCACAGCCTAAACCATC
LPAR3	ACGGTGATGACTGTCTTAG	TTGTAGGAGTAGATGATGGG
LPAR4	AAATATGCACTTCCAAAGGG	GGAAATATTTTCCTCCCCAAG
LPAR5	AATAATGTCACCACACACAC	GTTCTCAAAGTGTGATCCAG
LPAR6	ACCAAGAATTGTGAGAAAGC	TTCCGAAATAAACTCCCAAG

3.7 ELISA

Supernatants from melanoma cell cultures were collected after 12 h of 10 μM LPA treatment, and IL-10 concentration was quantified using the Human IL-10 ELISA kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

3.8 Flow Cytometry

Cells were washed and resuspended in PBS supplemented with 1% bovine serum albumin and stained with a DR6 (7678R, Bioss, Woburn, MA, USA) or HLA-DR (LN3, Invitrogen) antibody at 4 ºC for 30 min. At least 2 × 104 events per sample were counted by using flow cytometry (CytoFLEX, Beckman Coulter Life Sciences; Indianapolis, USA). Data were analyzed with the CytExpertCell software (Beckman Coulter Life Science).

3.9 Analysis of gene expression in melanoma samples

A transcriptomic database of immunotherapy-treated patient samples was established as described previously (120). The gene expression data was quantile normalized to integrate datasets generated using different technologies. From the entire database, only samples from patients treated with anti-PD-1 therapies, specifically nivolumab or pembrolizumab, were included.

To increase the sample size and robustness of the analysis, we included all available patients, irrespective of tumor histology. However, only pre-treatment samples, those obtained before the initiation of immune therapy, were used to evaluate the correlation between LPAR1 expression and the effectiveness of anti-PD-1 therapy. This approach was taken to avoid the confounding effects of ongoing systemic immune modulation.

3.10 Statistical Analysis

Statistical analysis was performed using Prism 6 (GraphPad Software Inc.; La Jolla, CA, USA). All data are presented as mean ± SEM obtained from at least three independent experiments. Statistical

significance was analyzed using one-way ANOVA and Dunnett's post hoc test and was considered at $p < 0.05$.

For the correlation analysis, Spearman rank correlation was computed. To evaluate the correlation with therapy response, receiver operating characteristic (ROC) analysis was performed, and the area under the curve (AUC) value was calculated to determine the overall predictive effect.

4. Results

First, the effect of LPA on the expression of the DR6 was determined and found that LPA dose-dependently upregulates the transcript levels of DR6 in HEK293T cells (Figure 1A). Measuring luciferase activity in HEK293T cells transfected with a human DR6 promoter construct revealed that LPA increased the DR6 promoter activity within 30 minutes compared to the vehicle-treated control, and the endogenous expression of DR6 mRNA also increased with a similar time course (Figure 1B). However, while the promoter activity declined after 60 min, the mRNA level remained elevated even at 3 h after LPA stimulation.

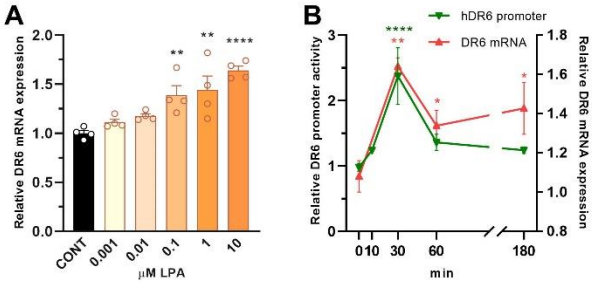

Figure 1. LPA-mediated dose and time-dependent upregulation of DR6 in HEK293T cells.

Our next aim was to identify the receptor mediating the effect of LPA. Of the six LPA GPCRs, LPAR1 and LPAR3 showed the highest expression levels in HEK293T cells (Figure 2A); therefore, we tested if inhibitors selective for either LPAR1/3 (Ki16425) or LPAR1 (AM095) can interfere with the effect of LPA. Both Ki16425 and AM095 were able to inhibit the effect of LPA on DR6 promoter activity (Figure 2B) and mRNA levels (Figure 2C), suggesting that LPAR1 is the receptor involved in mediating DR6 upregulation.

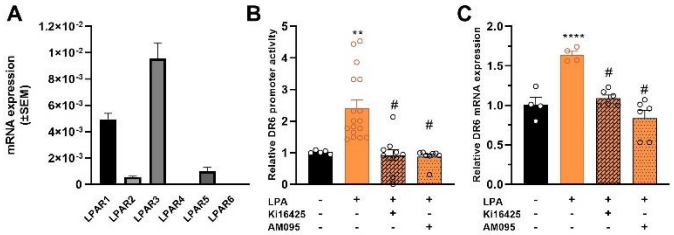

Figure 2. The identification of LPAR involvement in DR6 upregulation.

To explore the potential role of the LPA-DR6 axis in melanoma, we investigated the effect of LPA on DR6 expression in A375 (Figure 3A and B) and A2058 human melanoma cells (Figure 3C and D). LPA increased DR6 mRNA and protein levels in both melanoma cell lines. LPA-induced DR6 mRNA transcript levels reached a maximum at 1 h, whereas the expression of DR6 receptor on the cell surface peaked at 3 h in both melanoma cell lines. In the A375 cell line, LPA-induced DR6 protein expression was biphasic, with a second increase occurring at 12 hours (Figure 3B).

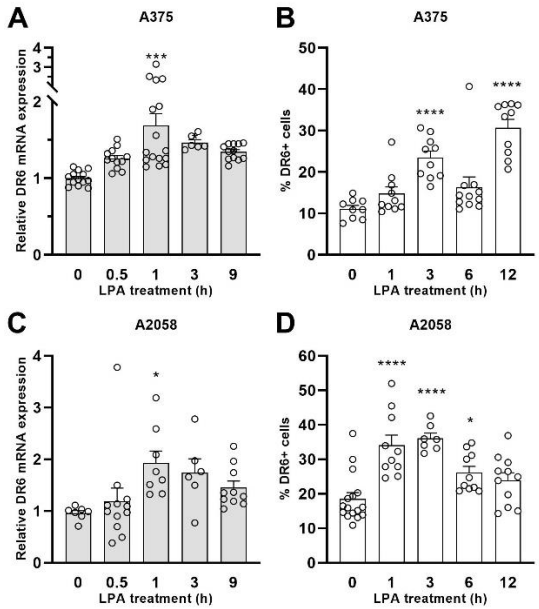

Figure 3. The time course of LPA-mediated DR6 expression in human melanoma cells.

Next, to elucidate the signaling pathways involved in LPA-induced DR6 upregulation, we analyzed the expression levels of different LPA receptors in A2058 and A375 melanoma cells and found that both express predominantly LPAR1 and LPAR3 receptors (Figure 4).

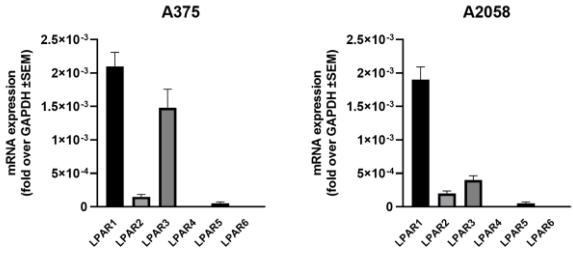

Figure 4. The LPAR mRNA profile of A375 and A2058 melanoma cells.

Inhibiting the LPAR1/3 receptors with Ki16425 or selectively LPAR1 using AM095 completely abolished LPA-induced DR6 mRNA expression in both cell lines, supporting a central role of LPAR1 in the process (Figure 5A and C). To further validate these findings, we used flow cytometry and showed that LPA-induced marked increase in the protein level of DR6 was inhibited by AM095 in both A375 (Figure 5B) and A2058 cells (Figure 5D).

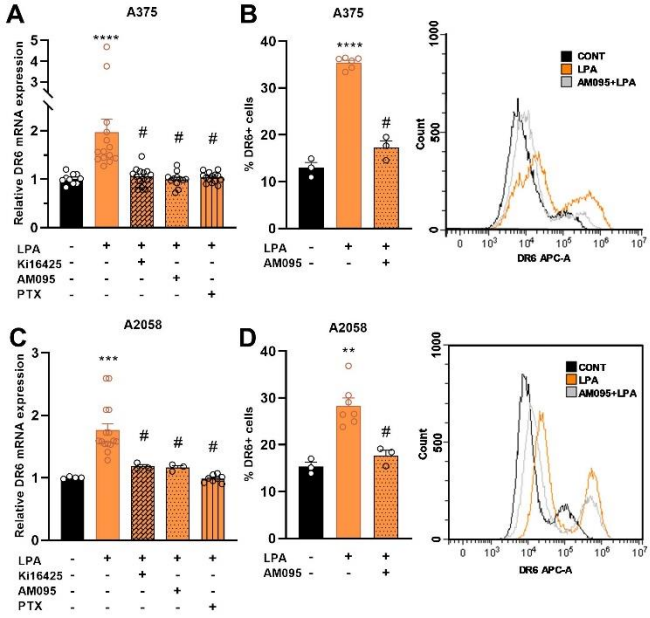

Figure 5. LPA induced DR6 upregulation via G_i-coupled LPAR1 in human melanoma cells.

To identify the G-protein involved in LPAR1-mediated upregulation of DR6, melanoma cells were pretreated with pertussis toxin (PTX). PTX, a specific inhibitor of G_i, abrogated the effect of LPA in both melanoma cell lines (Figure 5A and C).

Next, we aimed to analyze the transcriptional regulation of DR6 expression. Using the ALGGEN-PROMO software, we found that the

putative promoter sequence of DR6 contains binding sites for the transcription factor (TF) NF-κB1 (Figure 6A). Since LPA is a known activator of NF-κB, we investigated its involvement in LPA-induced DR6 expression. We showed that siRNA silencing of NF-κB1 abrogated the LPA-induced DR6 expression without affecting basal DR6 expression (Figure 6B). Our results indicate that stimulation of the G_i-coupled-LPAR1 by LPA increases DR6 expression via activation of NF-κB1 in both A2058 and A375 melanoma cell lines.

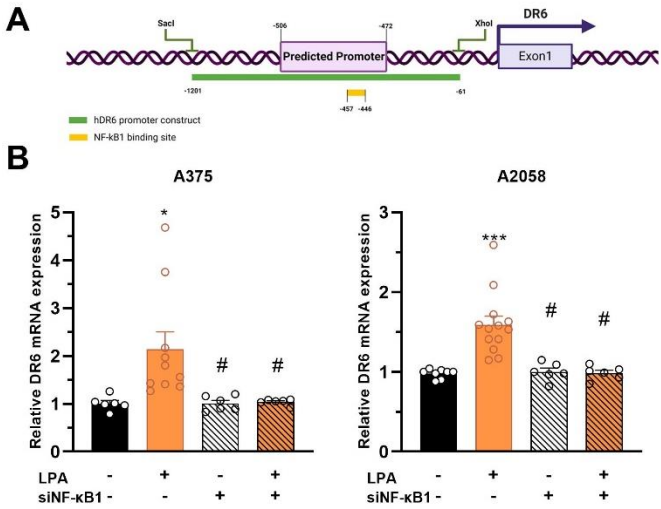

Figure 6. Identification of the transcription factor NF-κB1 in LPA-mediated DR6 expression.

As IL-10 has been reported to play a crucial role in melanoma progression, we investigated whether LPA could regulate IL-10 expression. We found that LPA increased IL-10 transcript with a similar time course in A2058 and A375 melanoma cells, resulting in maximal mRNA expression at 3h (Figure 7A and C). Using AM095 and PTX shown that LPA-induced upregulation of IL-10 is mediated via the LPAR1-G_i pathway (Figure 7B and D).

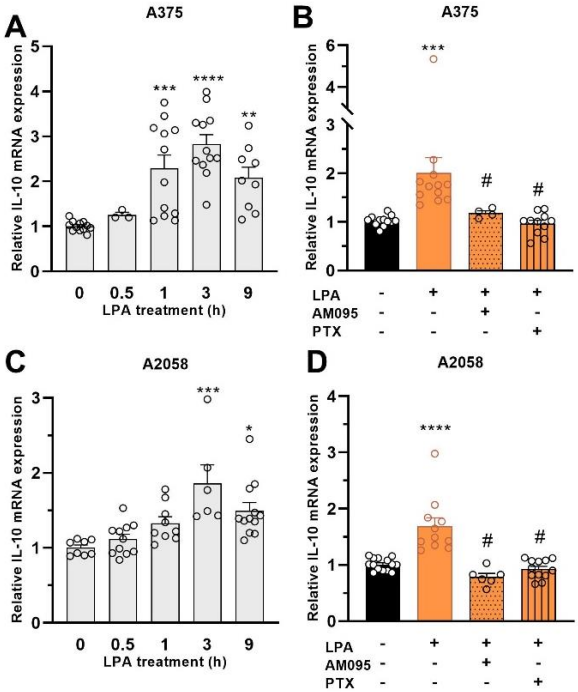

Figure 7. LPA mediated IL-10 expression via G_i-coupled LPAR1.

As LPA-induced IL-10 expression appeared to be mediated by the same signaling steps that were previously identified in DR6 upregulation, we hypothesized that DR6 may be involved in the LPA-mediated IL-10 production. Therefore, we evaluated whether silencing the DR6 gene with siRNA interferes with LPA-induced IL-10 mRNA expression. Interestingly, blocking DR6 expression with siRNA abolished the LPA-induced increase of IL-10 mRNA levels without influencing those in the absence of LPA (Figure 8). These results indicate that DR6 is responsible for mediating LPA-induced IL-10 expression.

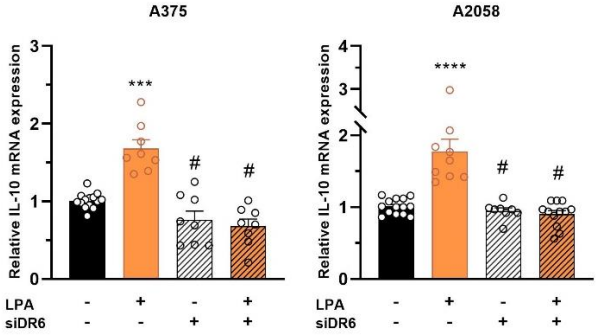

Figure 8. LPA-mediated IL-10 expression via DR6 in melanoma cells.

Moreover, we showed that the LPA-induced secretion of IL-10 in melanoma cells is mediated by LPAR1 and DR6. Specifically, LPA induced a 3- and 5-fold increase in IL-10 secretion of A375 and A2058 cells, respectively (Figure 9). These effects were abolished completely by pharmacological inhibition of LPAR1 or silencing of the DR6 gene (Figure 9), providing evidence for the involvement of the LPA-LPAR1-DR6 axis in increasing IL-10 secretion in melanoma.

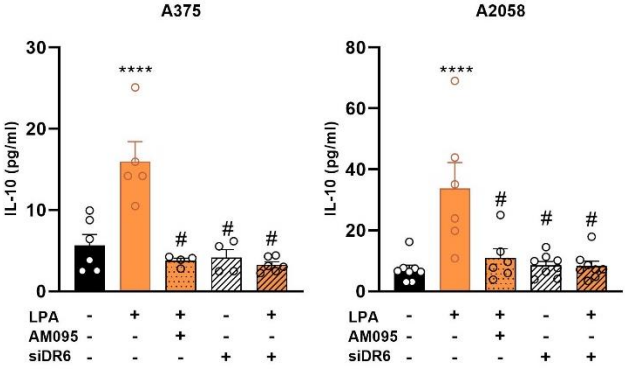

Figure 9. LPA-mediated IL-10 production in melanoma cells.

Databases of patients with melanoma were analyzed using Spearman rank correlation to verify the significance of the LPAR1−NF-κB1−DR6−IL-10 signaling cascade *in vivo*. Based on 435 melanoma

samples, LPAR1 expression strongly correlates with NF-κB1 (Spearman's r=0.23, p=9.8×10-7), DR6 (Spearman's r=0.33, p=1.9×10-12) and IL-10 (Spearman's r=0.21, p=1.3×10-5) expression (Figure 10). Supporting our findings, not only LPAR1, but DR6 also positively correlates with IL-10 (Spearman's r=0.19, p=7.6×10-5) (Figure 10). These results are consistent with our *in vitro* findings on the LPAR1−DR6−IL-10 signaling cascade in human melanoma.

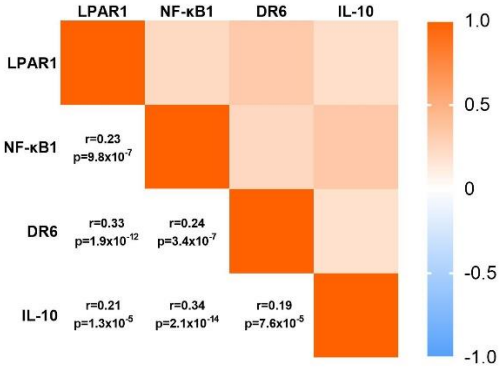

Figure 10. Correlation between the expression of LPAR1, NF-κB1, DR6 and IL-10 in melanoma tumor samples.

Next, we investigated whether the LPA-LPAR1-DR6 axis and subsequent IL-10 release affect HLA-DR expression in human melanoma cells. To do this, A375 or A2058 cells were treated with LPA in the absence and presence of the LPAR1 antagonist AM095, siRNA silencing of DR6 or IL-10, as well as in the presence of anti-IL-10 neutralizing monoclonal antibody or isotype control IgG1 kappa. Treatment with LPA alone resulted in a marked downregulation of HLA-DR in both A375 and A2058 melanoma cells which disappeared after pharmacological inhibition of LPAR1 by AM095 or silencing DR6 expression by siDR6 (Figure 11).

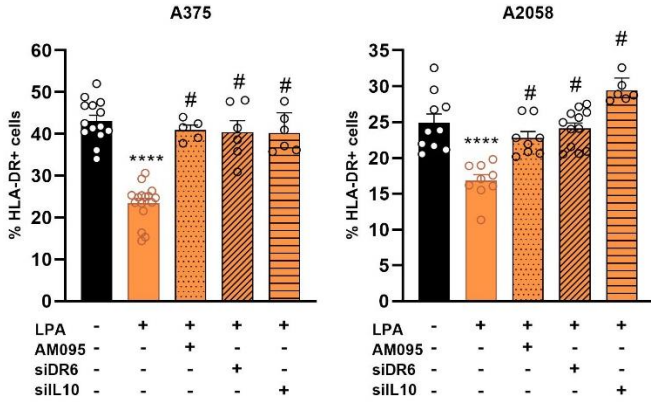

Figure 11. LPA-induced HLA-DR downregulation in melanoma cells.

More importantly, silencing IL-10 expression by siRNA (Figure 11) or blocking its effect by neutralizing antibody (Figure 12) completely abolished the effect of LPA on HLA-DR expression.

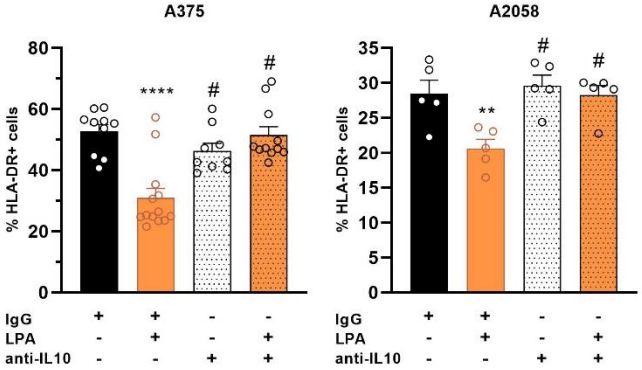

Figure 12. Neutralizing IL-10 inhibited the effect of LPA on HLA-DR expression.

These results revealed that LPA downregulates HLA-DR expression via activating the LPAR1-DR6-IL-10 pathway in both human melanoma cell lines.

Investigating the gene expression profile associated with resistance to anti-PD-1 therapy in melanoma tumors revealed that LPAR1 expression is significantly higher in non-responders compared to the responder group, confirming the marked role of LPAR1 in melanoma progression. This analysis highlights the potential predictive value of LPAR1 expression in determining the response to anti-PD-1 therapy in melanoma patients. The findings suggest that increased expression levels of LPAR1 may be associated with worse therapeutic outcomes, underscoring the importance of further investigating this gene as a potential biomarker for immunotherapy response.

5. Conclusions

In the current study, we aimed to determine the role of LPA-DR6 axis in human melanoma immune escape and clarify the underlying mechanisms. Our findings indicate that:

- LPA increases DR6 promoter activity and DR6 mRNA levels within 30 minutes, implying that DR6 acts as an immediate early response gene to LPA.
- Ki16425 or AM095 blocks the LPA-induced increase of DR6 promoter activity and expression, indicating that LPAR1 mediates this effect.
- LPA regulates DR6 mRNA and protein levels in A375 and A2058 human melanoma cells via LPAR1, in A375 cells with a biphasic increase in DR6 protein expression with a second peak at 12 hours.
- Pertussis toxin (PTX), a Gi protein inhibitor, abrogates the effect of LPA, indicating a Gi pathway involvement.
- The transcription factor NF-κB1 is crucial for LPA-induced DR6 expression, as its silencing negates the effect without altering basal DR6 expression.
- LPA increases IL-10 mRNA levels via LPAR1-Gi pathway in A375 and A2058 melanoma cells.

- DR6 silencing negotiates the LPA-induced increase in IL-10 transcription and secretion in human melanoma cells.

- In human melanoma samples LPAR1 expression positively correlates with NF-κB1, DR6, and IL-10 expression, confirming the in vitro observations.

- LPA downregulates HLA-DR expression in A375 and A2058 melanoma cells via the LPAR1-DR6-IL-10 pathway, highlighting a potential autocrine mechanism of anti-PD-1 immune checkpoint blockade therapy resistance in melanoma.

- Elevated LPAR1 expression is associated with poor response to anti-PD-1 therapy in melanoma patients, considering it a predictive biomarker for immunotherapy outcomes.

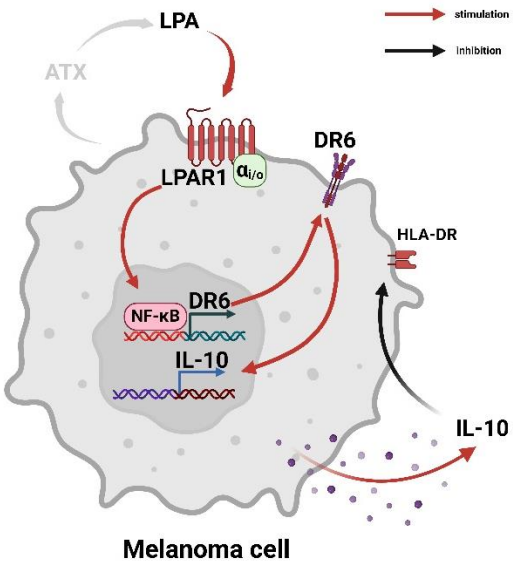

Figure 13. LPA, via its G_i-coupled LPAR1 receptor, activates NF-κB1-mediated DR6 expression, inducing the IL-10 which in turn leads to the downregulation of HLA-DR antigen in human melanoma cells. (Created with BioRender.com).

6. Bibliography of the candidate's publications

Related to the thesis:

Major E, Lin KH, Lee SC, Káldi K, Győrffy B, Tigyi G, Benyó Z. LPA suppresses HLA-DR expression in melanoma cells: a potential immune escape mechanism involving LPAR1 and DR6 receptor-mediated release of IL-10. Acta Pharmacologica Sinica. 2024 Aug 26.; **IF:6.9**

Not related to the thesis:

Besztercei B, Vancsik T, Benedek A, **Major E**, Thomas MJ, Schvarcz CA, Krenács T, Benyó Z, Balogh A. Stress-Induced, p53-Mediated Tumor Growth Inhibition of Melanoma by Modulated Electrohyperthermia in Mouse Models without Major Immunogenic Effects. International Journal of Molecular Sciences. 2019 Aug 17;20(16):4019. **IF: 4.6**

Thomas MJ, **Major E**, Benedek A, Horváth I, Máthé D, Bergmann R, Szász AM, Krenács T, Benyó Z. Suppression of Metastatic Melanoma Growth in Lung by Modulated Electro-Hyperthermia Monitored by a Minimally Invasive Heat Stress Testing Approach in Mice. Cancers (Basel). 2020 Dec 21;12(12):3872. **IF: 6.6**

Giunashvili N, Thomas JM, Schvarcz CA, Viana PHL, Aloss K, Bokhari SMZ, Koós Z, Bócsi D, **Major E**, Balogh A, Benyó Z, Hamar P. Enhancing therapeutic efficacy in triple-negative breast cancer and melanoma: synergistic effects of modulated electro-hyperthermia (mEHT) with NSAIDs especially COX-2 inhibition in in vivo models. Mol Oncol. 2024 Apr;18(4):1012-1030. **IF: 6.6**

Major E, Benedek A, Szász MA, Benyó Z, Balogh A. Modulált elektro-hipertermia sejtpusztító hatásának mechanizmusa és kemoterápiával való kölcsönhatása B16F10 melanóma-sejtvonalon Magyar Onkológia. 2021 Mar 17;65(1):71-77.

Dank M, Balogh A, Benedek A, Besztercei B, Danics L, Forika G, Garay T, Hamar P, Karászi Á, Kaucsár T, Kiss É, Krenács T, **Major**

E, Mohácsi R, Portörõ I, Ruisanchez É, Schvarcz C, Szász MA, Mbuotidem TJ, Vancsik T, Zolcsák Z, Benyó Z. Elektromágneses daganatterápiás készülék preklinikai és klinikai vizsgálatai, valamint mûszaki továbbfejlesztése: tapasztalatok szolid tumorokkal [Preclinical and clinical investigation and development of electromagnetic oncological device - experience with solid tumors]. Magyar Onkológia. 2019 Dec 9;63(4):354-358.

Cumulative impact factor of the candidate's publications: **23.1**