DOI:10.14753/SE.2025.3109

SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

3109.

MAJOR ENIKŐ

Kórélettan és transzlációs medicina
című program

Program- és témavezető: Dr. Benyó Zoltán, egyetemi tanár

DOI:10.14753/SE.2025.3109

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

PhD thesis

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Budapest
2024

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Abbreviations

AC	Adenylate Cyclase
AKT	Protein kinase B
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ATX	Autotaxin
AUC	Area Under Curve
BCL-2	B-Cell Lymphoma-2
BCL-xL	B-Cel Lymphoma-extra Large
BRAF	B-Raf proto-oncogene
BSA	Bovine Serum Albumin
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T lymphocyte Antigen-4
DC	Dendritic Cell
DR6	Death Receptor 6
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
ENPP2	Ectonucleotide Pyrophosphatase/Phosphodiesterase 2
ERK	Extracellular signal-Regulated Kinase
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate

GPCR	G Protein-Coupled Receptor
HLA-DR	Human Leukocyte Antigen DR subtype
ICAM-1	Intracellular Adhesion Molecule 1
ICI	Immune Checkpoint Inhibitor
IFN-γ	Interferon gamma
IL-10	Interleukin 10
LPA	Lysophosphatidic Acid
LPAR1	LPA G protein-coupled receptor subtype 1
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin-1 Receptor
MDM2	Mouse Double Minute 2 homolog
MEK	Mitogen-activated protein
MHC	Major Histocompatibility Complex
mTOR	Mammalian Target of Rapamycin
NF1	Neurofibromin 1
NF-κB	Nuclear Factor kappa B
NK	Natural Killer
NRAS	Neuroblastoma Ras viral oncogene homolog
PD-1	Programmed Death Receptor 1
PD-L1	Programmed Death Receptor Ligand 1
PI3K	Phosphoinositide 3-Kinase
PLC	Phospholipase C

PTEN	Phosphatase and Tensin homolog
PTX	Pertussis Toxin
qPCR	Quantitative Real-Time Polymerase Chain Reaction
Raf	Rapidly Accelerated Fibrosarcoma
Ras	Rat sarcoma virus
Rho	Ras homologous
ROC	Receiver Operating Characteristic
STAT3	Signal Transducer and Activator of Transcription 3
TAA	Tumor Associated Antigen
TCR	T Cell Receptor
TLR	Toll Like Receptor
TGF-β	Transforming Growth Factor beta
TNF-α	Tumor Necrosis Factor alpha
TNFRSF21	Tumor Necrosis Factor Receptor Superfamily 21
TME	Tumor Microenvironment
TP53	Tumor Protein p53
Treg	Regulatory T cell
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor

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1. Introduction

1.1 Lysophosphatidic acid

Lysophosphatidic acid (LPA), a bioactive lipid mediator, was first identified over three decades ago and has since exhibited medicinal relevance. In the plasma, lysophosphatidylcholine (LPC) and other lysophospholipids are the primary sources of LPA, which is generated by the lysophospholipase D enzyme, autotaxin (ATX, encoded by the human *ENPP2* gene) (1,2). Interestingly, ATX was first identified in A2058 human melanoma cells as an autocrine motility factor (3), and subsequent analysis revealed that the compound was identical to plasma lysophospholipase D, which is responsible for converting LPC to LPA (4). ATX is widely expressed, with the highest mRNA levels observed in the brain, lymph nodes, kidney, and testis (5). Additionally, it is overexpressed in various cancers.

LPA levels are significantly increased in both plasma and tumor tissues in human cancers, indicating that LPA functions as a key factor in tumor development (6). Clinical studies revealed that high expression of ATX is linked to numerous malignancies, resulting in an elevated level of LPA, which contributes to both tumor progression and metastasis formation (7–9). Furthermore, it inhibits anti-tumor immunity in melanoma (6). The cellular communication within the tumor microenvironment (TME) is driven by a complex network of cytokines, enzymes, and lipids. Recent studies indicate that alterations in the lysophospholipid profile within the TME may represent a strategy used by tumor cells to circumvent the anti-cancer immune response (10).

LPA mediates multiple physiological and pathological functions through activation of its six confirmed G protein-coupled receptors, named LPAR1-6, with broad tissue distributions and overlapping signaling pathways **(Figure 1)** (11,12). Due to the widespread expression of LPA receptors and their connection to diverse G protein-mediated pathways, including $G_{i/o}$, G_s, $G_{q/11,}$ and $G_{12/13}$, LPA has been implicated in different physiological functions and disorders, such as vascular development, neurite remodeling, inflammation, and tumor progression (11,13,14). LPA receptors represent a promising avenue for therapeutic interventions, as evidenced by the extensive literature on LPA-mediated signaling in cancer development (5,15).

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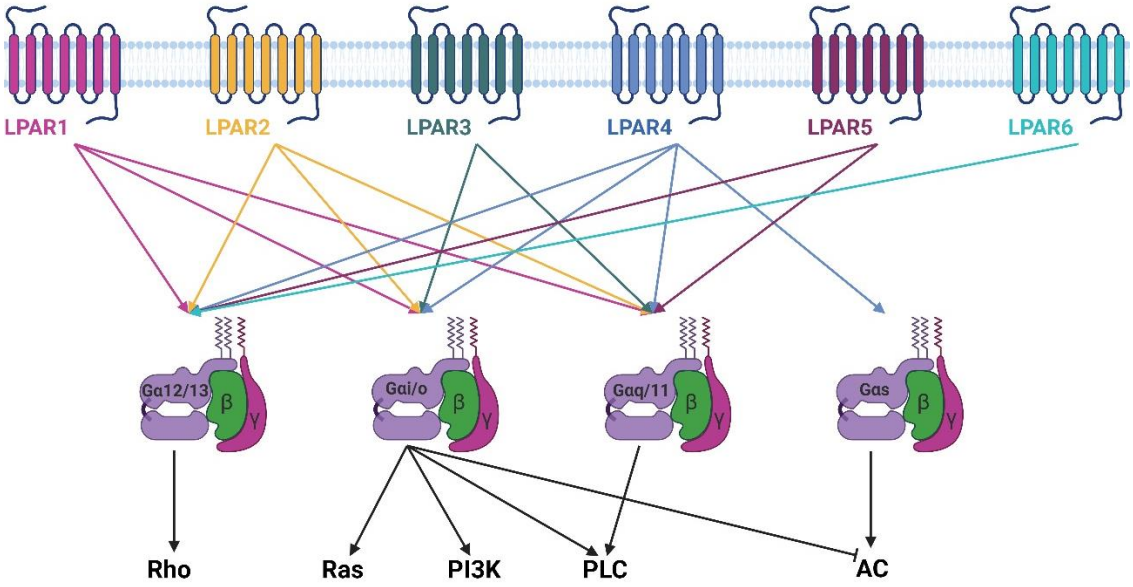

Figure 1. Signaling pathways of the G protein-coupled LPA receptors (12). LPA can induce numerous intracellular signaling pathway by activating its G-protein coupled receptors. AC- adenylate cyclase, PLC- Phospholipase C, PI3K- Phosphoinositide 3-kinase, Ras- Rat sarcoma virus, Rho- Ras homologous. (Created with BioRender.com)

The ATX-LPA-LPAR signaling axis was becoming one of the most studied fields in tumor biology due to its well-documented oncogenic potential. LPA reportedly mediates proliferation, survival, migration, angiogenesis, metastasis, and inflammation in carcinomas (16). Notably, it was observed in ovarian and gastric cancer cells that LPA regulates the cell cycle via LPAR1-, LPAR3-related EGFR/PI3K pathways (17,18). LPARs are able to activate a variety of signaling effectors, but overall, LPARs facilitate cancer cell proliferation and survival through $G_{q/11}$, G_i, and/or $G_{12/13}$ and predominantly enhance cell migration and invasion via G_i and/or $G_{12/13}$ (13,14,19).

1.2 Death receptor 6

Death receptor 6 (DR6, encoded by the human *TNFRSF21* gene) is a type I transmembrane receptor that belongs to the tumor necrosis factor superfamily (20). DR6 mRNA is expressed in various organs, including the brain, heart, pancreas, placenta, and immune organs such as lymph nodes and bone marrow (20,21). Since elevated DR6 expression has been found to play a pivotal role in human pathological conditions, including Alzheimer's disease, inflammatory processes, and autoimmune disorders, it has been identified as a promising target for therapeutic intervention (21,22). Compared to

normal tissue, abundant transcript levels of DR6 were observed in certain human cancers, suggesting its role in tumor biology (20,23–25). Likewise, studies demonstrated that upregulation of DR6 promotes tumor aggressiveness in lung cancer, (26), whereas low DR6 expression provides a higher overall survival probability in pancreatic adenocarcinoma (27). Furthermore, Yang et al. revealed that DR6 is required for tumor angiogenesis through inducing IL-6 production via NF-κB-dependent signaling in B16 murine melanoma (28).

DR6 has also been implicated in cell death signaling (29). DR6-mediated apoptosis is cell type-dependent, suggesting that its cellular signaling pathways could be very different from other death receptors (29). Interestingly, Dong et al. demonstrated that LPA is able to induce apoptosis via the upregulation of DR6 in HeLa cells (30), although other investigators did not confirm this effect under the same conditions (31,32).

1.3 Melanoma

Melanoma is a hazardous, deadliest type of malignant skin cancer. Its incidence is still increasing in the Western world with severe impacts on the life quality and expectancy of patients (33). Melanoma is a form of aggressive skin cancer arising from melanocytes (34). Melanocytes produce melanin, which gives normally the pigmentation of the skin, hair, and eyes as well as protection against solar radiation (34–36). A neoplastic transformation of the melanocytes derived from the neural crest is thought to be responsible for melanoma (37,38). It can occur anywhere on the body, although its location appears to be influenced by the age and sex of the patients (37). Melanoma can be distinguished as epithelial or non-epithelial origin, with benign, borderline, or malignant progression (39). In malignant tumors originating from epithelial-associated melanocytes, we distinguish familial, UV-induced, and non-UV-induced, and within the latter, mucosal, acro lentiginous, and uveal forms. UV-induced melanomas can be divided into chronic (chronic sun-induced damage, CSD) and intermittent (non-CSD) forms based on the duration of UV exposure (40).

However, the appearance of the tumor is inhomogeneous, and pathological changes in moles can develop in any area of the body, predominantly in areas exposed to direct UV radiation, such as the head, hands, and feet (36–38). Around 20% of melanomas in the head and neck have a worse prognosis compared to those in other regions.

DOI:10.14753/SE.2025.3109

In the last three decades, recognizing leading gene defects has enabled the molecular diagnosis of malignant melanoma. These mutations primarily affect the PI3K/AKT and RAS/RAF/MEK/ERK signaling pathways (35,36,39) including the alteration of B-Raf proto-oncogene (BRAF), neurofibromin 1 (NF1), NRAS, phosphatase and tensin homolog (PTEN), and tumor protein p53 (TP53) **(Figure 2)**. Furthermore, Melanocortin-1 receptor (MC1R) is the most important melanocyte receptor, associated with pigmentation and protection against melanoma (39–41).

NF1, a tumor suppressor gene, is the third most common mutation, altered in 10%-15% of melanomas (37). NF1 inhibits RAS signaling by converting active RAS-GTP to inactive RAS-GDP (42). Loss of NF1 function results in NRAS hyperactivation and enhanced PI3K and MAPK pathway signaling **(Figure 2)**, commonly seen in melanomas associated with sun exposure, often with other genomic mutations, including NRAS and BRAF (43).

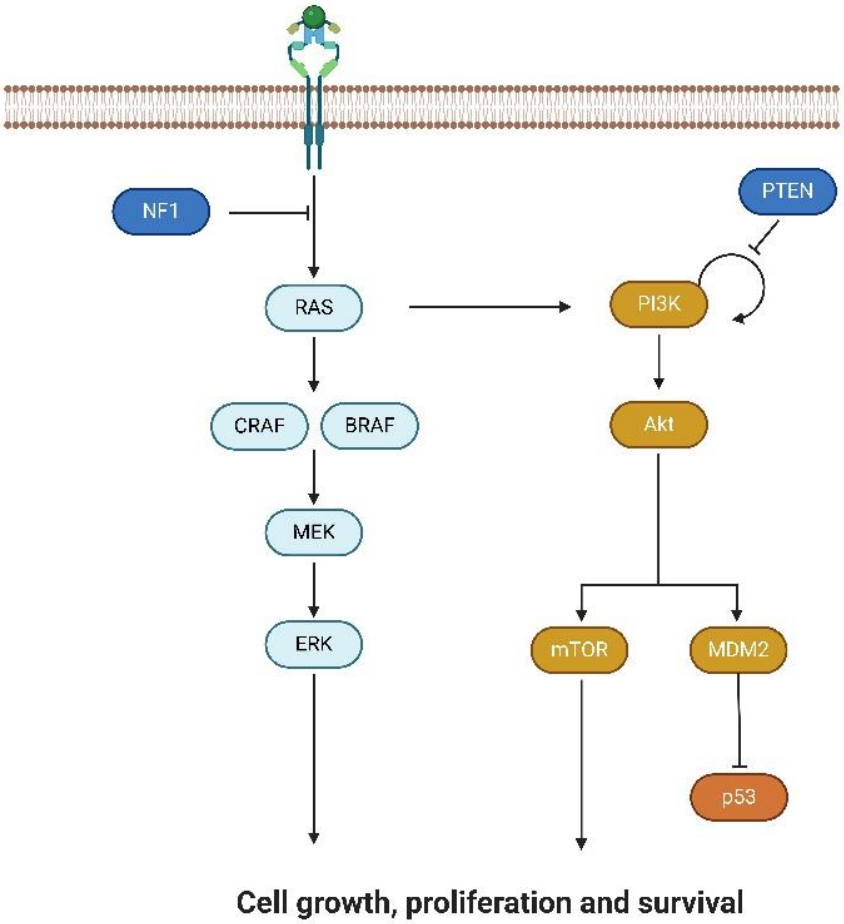

Figure 2. Dysregulated oncogenic signaling pathways in melanoma (44). AKT- Protein kinase B, BRAF- B-Raf proto-oncogene, ERK- Extracellular signal-regulated kinase,

MDM2- Mouse double minute 2 homolog, MEK- Mitogen-activated protein, mTOR- Mammalian target of rapamycin, NF1- Neurofibromin 1, PI3K- Phosphoinositide 3-kinase, PTEN- Phosphatase and tensin homolog, RAS- Rat sarcoma virus.(Created with BioRender.com)

NRAS activating mutations are the second most common in melanoma, present in 15%-30% of the cases (38,45,46). These mutations, mainly missense in codons 12, 13, or 61, prolong the active GTP-bound state of NRAS, leading to sustained aberrant signaling through the PI3K and MAPK pathways (46,47). BRAF and NRAS mutations are usually mutually exclusive but can co-occur occasionally.

BRAF is a proto-oncogene coding for a serine/threonine-protein kinase crucial in cell growth and proliferation, mainly via the RAS-RAF-MEK-ERK pathway (37,38,48). Activating BRAF mutations, particularly the V600E, which converts valine to glutamate, are present in 60% of melanomas and lead to uncontrolled cell proliferation and tumor development (37,48). More importantly, 60%-70% of advanced melanomas have BRAF mutations, indicating their role in progression. These mutations are more frequently observed in younger patients with intermittent sun exposure compared to those with chronic sun exposure (48). Melanomas with BRAF mutations represent distinctive characteristics, including increased aggressiveness compared to BRAF wild-type (WT) melanomas, a higher tendency to metastasize to the brain, and a correlation with reduced survival in patients with stage IV tumors (49). Due to their prevalence and resistance to targeted therapy, BRAF V600 mutations are important targets in melanoma treatments, with many drugs developed to reverse the consequences of this mutation (37,48,49).

PTEN is a tumor suppressor gene that regulates the cell cycle. Dysregulation of PTEN occurs in 10%-30% of cutaneous melanoma cases, mainly in the vertical growth phase and metastases (37,49,50). Common PTEN alterations include missense and frameshift mutations, chromosomal deletions, and epigenetic mechanisms such as microRNAs. PTEN mutations often co-occur with BRAF mutations but are mutually exclusive with NRAS mutations (51). Loss of PTEN function leads to increased PI3K/AKT pathway activation **(Figure 2)**, contributing to melanoma progression and resistance to BRAF inhibitors (37, 50,52). Furthermore, Cabrita et al. revealed that PTEN alterations promote immune evasion by influencing the cytotoxic T-cell signal within the tumor (52).

Ultraviolet (UV) light radiation from sunlight is a significant environmental factor in melanoma development (41,53). Sunlight, especially intermittent exposure to high intensity, increases melanoma risk as the UV-B spectrum penetrates the epidermis, targeting melanocytes (53). A history of sunburn, especially in childhood, significantly raises melanoma risk, as does exposure to artificial UV-A sources like indoor tanning devices (54). UV radiation has been classified as a human carcinogen (54). Host factors such as the frequency and type of melanocytic nevi, genetic predisposition, and family history also influence melanoma risk (37). Melanomas often develop on pre-existing nevi. Genetic factors, including polymorphisms in the MC1R gene, contribute to skin color phenotypes and UV sensitivity, with light-skinned individuals being more vulnerable (37,41). Certain genetic disorders like familial atypical multiple mole-melanoma syndrome, melanoma-astrocytoma syndrome, familial retinoblastoma, Xeroderma pigmentosum are linked to a higher melanoma risk (37).

Melanoma exhibits resistance to hypoxic conditions, oxidative stress, and apoptosis-inducing processes, which may promote the formation of metastases (55,56). Since melanoma is often visible on the skin surface, early diagnosis and surgical excision are possible (stage I-II). However, if it is not recognized in time, it metastasizes first in the surrounding lymph nodes (stage III), in which the 5-year survival rate is 60%, depending on the size of the primary tumor and the extent of its spread in the surrounding lymph nodes and organs. If the melanoma has spread to more distant lymph nodes and organs (stage IV), the 5-year survival rate is decreased to 16%, and the treatment is more complicated (57). In many cases, if surgical excision is not possible or ineffective alone, other adjuvant treatments such as chemotherapy, targeted therapy, immunotherapy, and their combination are used.

1.4 Immunotherapy in melanoma

Extensive research has been conducted on the tumor microenvironment and the role of different immune cells in tumor therapy, developing antitumor treatments to re-activate the host’s immune system. As melanoma is one of the most immunogenic malignant cancer types (58), immunotherapy has changed the life expectancy of patients with this aggressive skin cancer over the last decade (59,60).

It is known that tumors have different strategies to suppress the host immune response. Inhibiting these strategies enables the immune system to be re-activated, resulting in effective antitumor treatment. The cycle of tumor immunity consists of two main phases: an initial nonspecific phase activating innate immunity through macrophages, granulocytes, dendritic cells (DCs), and natural killer (NK) cells, followed by a later phase where effector CD4+ and CD8+ T-cells target melanoma cells (59). This targeting is facilitated by interferon-gamma production and involves direct tumor cell cytotoxicity via MHC-TCR interactions or antigen-specific T-cell activity during the adaptive immune response. The initial event that enables melanoma cells to evade the immune system is the defective immune recognition of tumor-specific antigens. This is caused by inefficient antigen processing, which incrementally impairs the ability of CD8+ T cells to recognize and respond to the processed antigens expressed by tumor cells (59). Furthermore, the efficacy of T-cell cytotoxicity is determined by the appropriate presentation of antigens by antigen-presenting cells (APCs). The activity, co-stimulation, and antigen presentation by these cells are critical for the induction of functional immunity. The maturation and priming of DCs are influenced by stimuli from their microenvironment, where factors such as VEGF, IL-8, and IL-10 produced by melanoma cells promote an immature phenotype (59). Moreover, impairment of DCs is associated with diminished co-stimulation activity, which can be attributed to the inadequate expression of CD80 and CD86. Lytic cell death of tumor cells is associated with CD8+ cells, activated by tumor-associated antigens (TAA), which lyse target cells by releasing granules containing perforin and granzyme B (61).

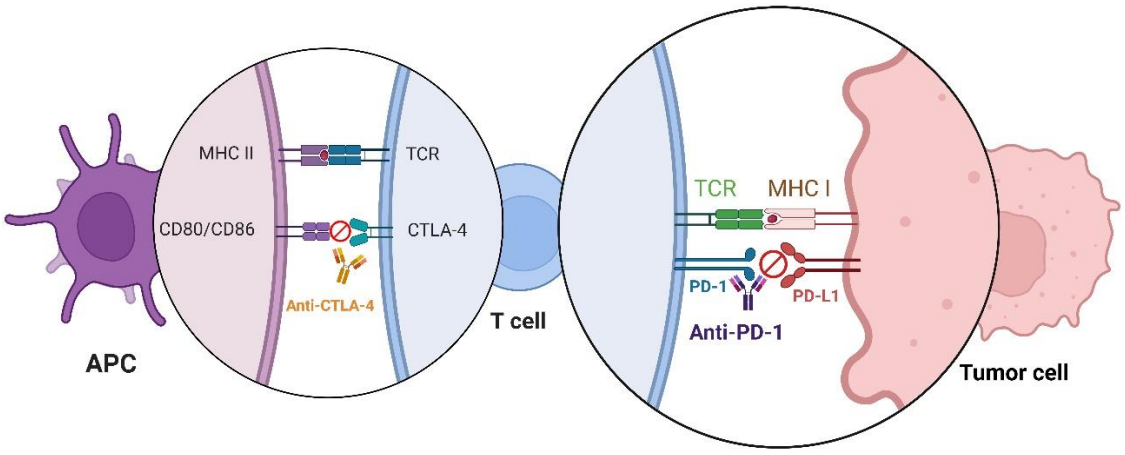

Figure 3. Targets of anti-CTLA-4 and anti-PD-1 immune checkpoint blockade (62,63). APC- Antigen presenting cell, CTLA-4- Cytotoxic T lymphocyte antigen-4, MHC- Major

Histocompatibility Complex I/II, TCR- T Cell Receptor, PD-1- Programmed Death Receptor 1, PD-L1- Programmed Death Receptor Ligand 1 (Created with BioRender.com)

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is a major co-receptor inhibitor in immune regulation, expressed by T cells binding to CD80/86 on the surface of the APC, causing inhibition of T cell activation **(Figure 3)** through suppressing the expression of interleukin 2 (IL-2) (64,65). This antigen has a vital role in limiting the effective immune response against tumor cells. Clinical trials of anti-CTLA-4 antibodies have demonstrated their efficacy in the treatment of melanoma (65,66). Two human anti-CTLA-4 monoclonal antibodies, ipilimumab and tremelimumab, are available (67). Ipilimumab is the most widely studied anti-CTLA-4 antibody and has been used successfully in the treatment of a variety of tumor types (68). In its evaluation, taking into account its immunotherapeutic effect (69), it has been shown to improve the clinical outcome (long-term stability, reduction in cancer progression) by 30% of patients with advanced stage IV melanoma who had failed prior targeted and/or chemotherapy (70). Phase III trials have shown significantly higher overall survival in a combined protocol with dacarbazine compared to dacarbazine alone (71–74). Its use both as a stand-alone therapy and as combined therapy in the treatment of stage III melanoma significantly increased overall survival (75,76). The other anti-CTLA-4 antibody, tremelimumab, has shown no difference in efficacy compared with conventional chemotherapeutic agents (77), but it has a much more severe immune side effect profile compared to ipilimumab (78).

The other important antitumor checkpoint is Programmed death receptor-1 (PD-1), an inhibitory receptor found on the surface of DC, B-, T- (CD4+ and CD8+) and NK cells (79). PD-1 has an important role in regulating the immune response, including its inhibitory effect on T cell proliferation. PD-L1 (programmed cell death receptor ligand-1) is found in increased amounts on the surface of many cells, such as epithelial cells, endothelial cells, T cells-, and tumor cells (80,81). PD-1:PD-L1 interaction prevents the activation of PI3K/Akt and MAPK/ERK pathways (81). Inhibition of this interaction by monoclonal antibodies can restore the cytotoxic function of T cells, enabling them to destroy tumor cells **(Figure 3)** (81). Various anti-PD-1 antibodies have been developed that have shown significant therapeutic effects with reduced toxicity compared to anti-CTLA-4 therapy in numerous types of tumors, including melanoma (82). The first anti-

PD-1 antibody was nivolumab, which has been shown to be more efficient compared to dacarbazine in clinical trials (83,84). More recently, a clinical trial combining nivolumab with ipilimumab has resulted in a significant increase in overall survival compared to ipilimumab monotherapy (85). Another anti-PD-1 antibody is pembrolizumab, which, like nivolumab, is used in the treatment of advanced melanoma patients previously treated with ipilimumab or a BRAF inhibitor, with increased overall survival compared to anti-CTLA-4 therapy (86).

Although immunotherapy extends many patients' life expectancy, clinical studies confirm that half of the patients do not respond to immunotherapy (87,88), which emphasizes the relevance of investigating the molecular mechanisms behind the ineffectiveness. Recently, numerous clinical studies identified that human leukocyte antigen DR (HLA-DR), an MHC class II antigen, correlates with the outcome of immune checkpoint inhibitor (ICI) treatment (87,89–92). HLA-DR is expressed mainly by professional antigen-presenting cells, but other cell types, including some tumors, are also able to express it (93).

1.5 Interleukin 10

The pleiotropic cytokine interleukin-10 (IL-10) is elevated in several malignancies and regulates the secretion of other cytokines (94). It was discovered in the late 1980s and was named cytokine synthesis inhibitory factor (95). Among the IL-10 family members, it has been recognized as a key member mediating different functions within the immune system and cancer cells (94,96). It is primarily recognized as an anti-inflammatory cytokine secreted by immune cells; however, it was later demonstrated that non-immune cell types, including fibroblasts and keratinocytes, and various types of tumor cells, such as breast, colon carcinoma, and melanoma cells, can also produce IL-10 (97). IL-10 plays a significant role in both innate and adaptive immunity. Its production is induced by various stimuli in different immune cells, primarily monocytes and T cells, but also in dendritic cells, B cells, natural killer cells, mast cells, neutrophils, and eosinophils (98). During infections, macrophages stimulated by Toll-like receptors (TLRs) are the main IL-10 producers (98,99). Likely B cells when activated by TLRs and IFN-α in combination with TLR agonists (98,100). However, Regulatory T cells (Tregs) influenced by IL-2, IL-4, and TGF-β, which are involved in a positive feedback loop, are

known as significant players in maintaining high IL-10 levels (94, 98,100). IL-2 and IL-27 induce IL-10 in CD8+ T cells, while IL-12 and IL-23 prime CD8+ and CD4+ T cells for IL-10 production (94,101,102). IL-10 has both immunosuppressive and immunostimulatory effects. It reduces malignant cell sensitivity to cytotoxic T cells but increases NK cell cytotoxicity, suggesting a role in combating malignancies by enhancing innate immune responses (94).

Its role in tumor development is the subject of ongoing debate, with suggestions that it may act either as a tumor suppressor or promoter. However, due to the complexity of IL-10 and the lack of consensus on its role in the TME, this field requires further investigation. Most of the existing literature suggests pro-tumoural activity of IL-10, with a focus on its impact in different oncological settings (94). IL-10 can contribute to cancer progression by activating Signal transducer and activator of transcription 3 (STAT3) signaling pathway, which upregulates BCL-2, BCL-xL, and cell proliferation factors like cyclins and c-Myc (103). Otherwise, its immunosuppressive activity on macrophages and DCs can lead to tumor immune evasion by reducing antigen presentation and cell maturation. High IL-10 levels correlate with shorter survival times in diffuse large-cell lymphoma and poor prognosis in peripheral T-cell lymphoma, indicating lower complete response rates and higher early relapse rates (104,105). Nonetheless, elevated IL-10 at diagnosis is an independent prognostic marker in adult hemophagocytic lymphohistiocytosis, aiding in treatment strategy decisions (106). Similarly, elevated serum IL-10 levels are reportedly associated with a poor prognosis in melanoma (107–110). Furthermore, abundant IL-10 expression is accompanied by an increase in other inflammatory mediators and worsens the outcomes of various cancers, indicating that IL-10 can be a key regulator of tumor immunity (111–115).

IL-10 plays a dual role, acting as pro-inflammatory and anti-inflammatory mediator (96,116). In cancer, IL-10, secreted by tumors or tumor-infiltrating immune cells can help malignant cells evade immune surveillance (117). However, its function is affected by various factors, including target cells, other stimuli (94). The complexity of IL-10 effects continues to prompt further research to understand its nature completely.

DOI:10.14753/SE.2025.3109

2. Objective

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 in order 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.

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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% CO_2. 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 G_i 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 $2^{-\Delta\Delta CT}$. The LPAR1-6 gene expressions were defined as $2^{-\Delta CT}$. 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 2×10^4 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.

DOI:10.14753/SE.2025.3109

4. Results

4.1 Effect of LPA on DR6 receptor expression in HEK293T cells

First, the effect of LPA on the expression of DR6 was determined, and it was found that LPA dose-dependently upregulates the transcript levels of DR6 in HEK293T cells **(Figure 4A)**. 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 4B)**. However, while the promoter activity declined after 60 min, the mRNA level remained elevated even at 3 h after LPA stimulation (121).

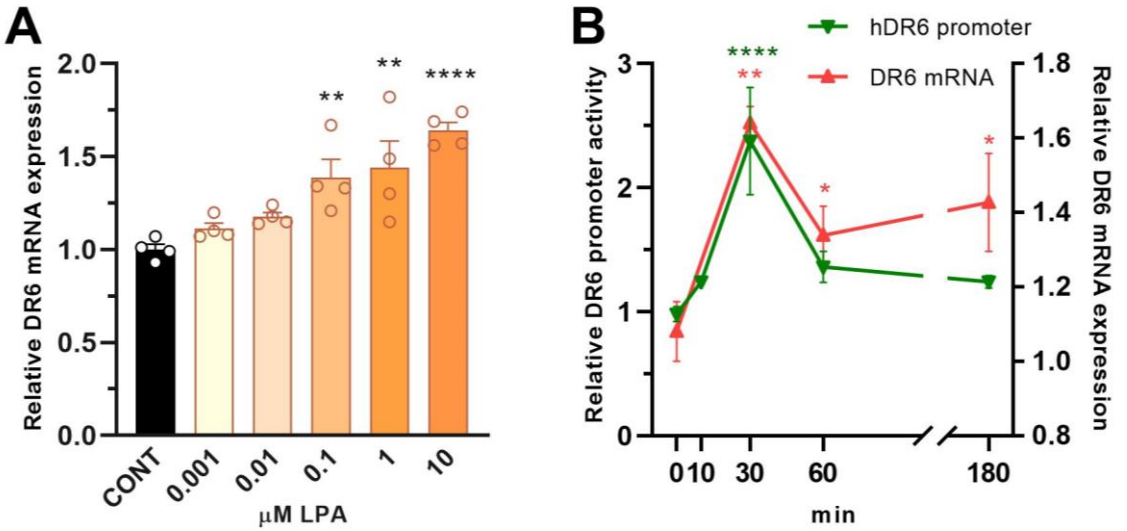

Figure 4. A. HEK293T cells were treated with the indicated concentrations of LPA for 30 min and DR6 expression was measured using qPCR. **B.** HEK293T cells transfected with the DR6 promoter construct were treated with 10 μM LPA for the indicated times and luciferase activity was measured (green line). Relative expression of the DR6 transcript was analyzed by qPCR (red line). All data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's posthoc test; *p<0.05, **p<0.01, ****p<0.0001. Figure adapted from the author's original publication (121).

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 5A)**; 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 5B)** and mRNA levels **(Figure 5C)**, suggesting that LPAR1 is the receptor involved in mediating DR6 upregulation (121).

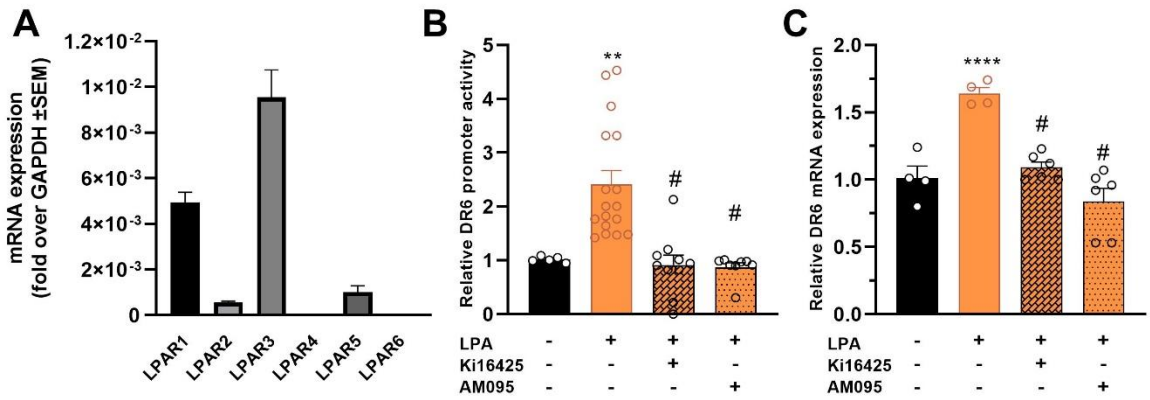

Figure 5. A. The LPAR mRNA expression profile of HEK293T cells. **B.** HEK293T cells transfected with DR6 promoter construct were treated with 10 µM LPA for 30 min. Where indicated, cells were pretreated with either LPAR1/3 inhibitor Ki16425 (10 µM) or LPAR1 inhibitor AM095 (10 µM). **C.** The inhibition of the LPA-induced upregulation of DR6 by Ki16425 or AM095 was confirmed by qPCR. All data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's posthoc test; $^{**}p<0.01$, $^{****}p<0.0001$ vs. control, $\#p<0.0001$ vs LPA alone. Figure adapted from the author's original publication (121).

4.2 Effect of LPA on DR6 expression in human melanoma cells

To explore the potential role of the LPA-DR6 axis in melanoma, we investigated the effect of LPA on DR6 expression in human A375 **(Figure 6A and B)** and A2058 melanoma cells **(Figure 6C and D)**. LPA treatment increased DR6 mRNA and protein levels in both melanoma cell lines. LPA-induced DR6 mRNA transcript levels reached a maximum at 1h, whereas the expression of DR6 receptor on the cell surface peaked at 3h after LPA treatment 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 6B)** (121).

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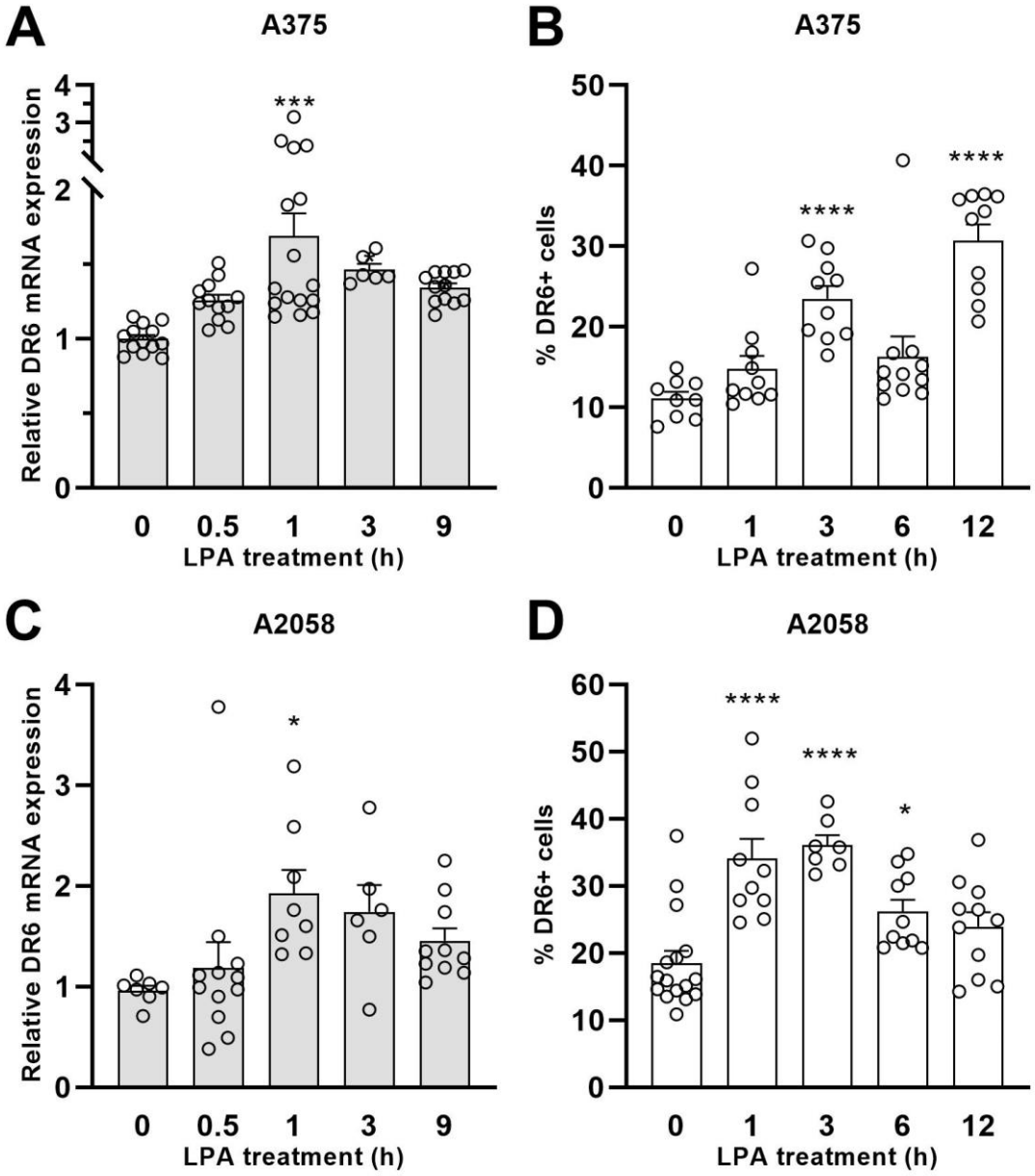

Figure 6. Melanoma cells were treated with 10 μM LPA. Time-dependent induction of DR6 expression was followed by qPCR (**A, C**) and flow cytometry (**B, D**). All data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's posthoc test; **p*<0.05, ****p*<0.001, *****p*<0.0001. Figure adapted from the author's original publication (121).

Next, with the aim of elucidating 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 7)** (121).

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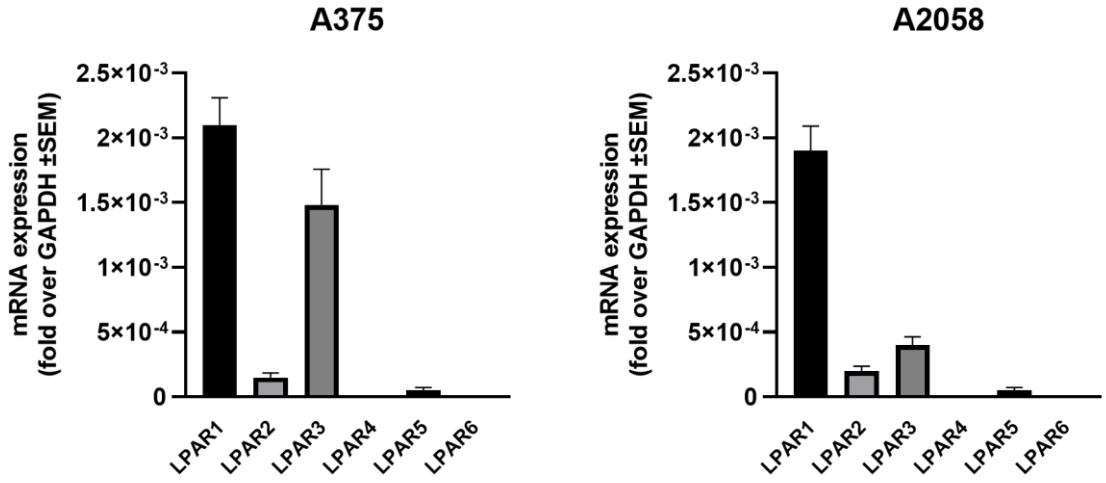

Figure 7. The mRNA profile of LPARs in A375 and A2058 melanoma cells was quantified by qPCR. Figure adapted from the author's original publication (121).

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 8A and C)**. To further validate these findings, we used flow cytometry and showed that LPA induced a marked increase in the protein level of DR6, which was inhibited by AM095 in both A375 **(Figure 8B)** and A2058 cells **(Figure 8D)** (121).

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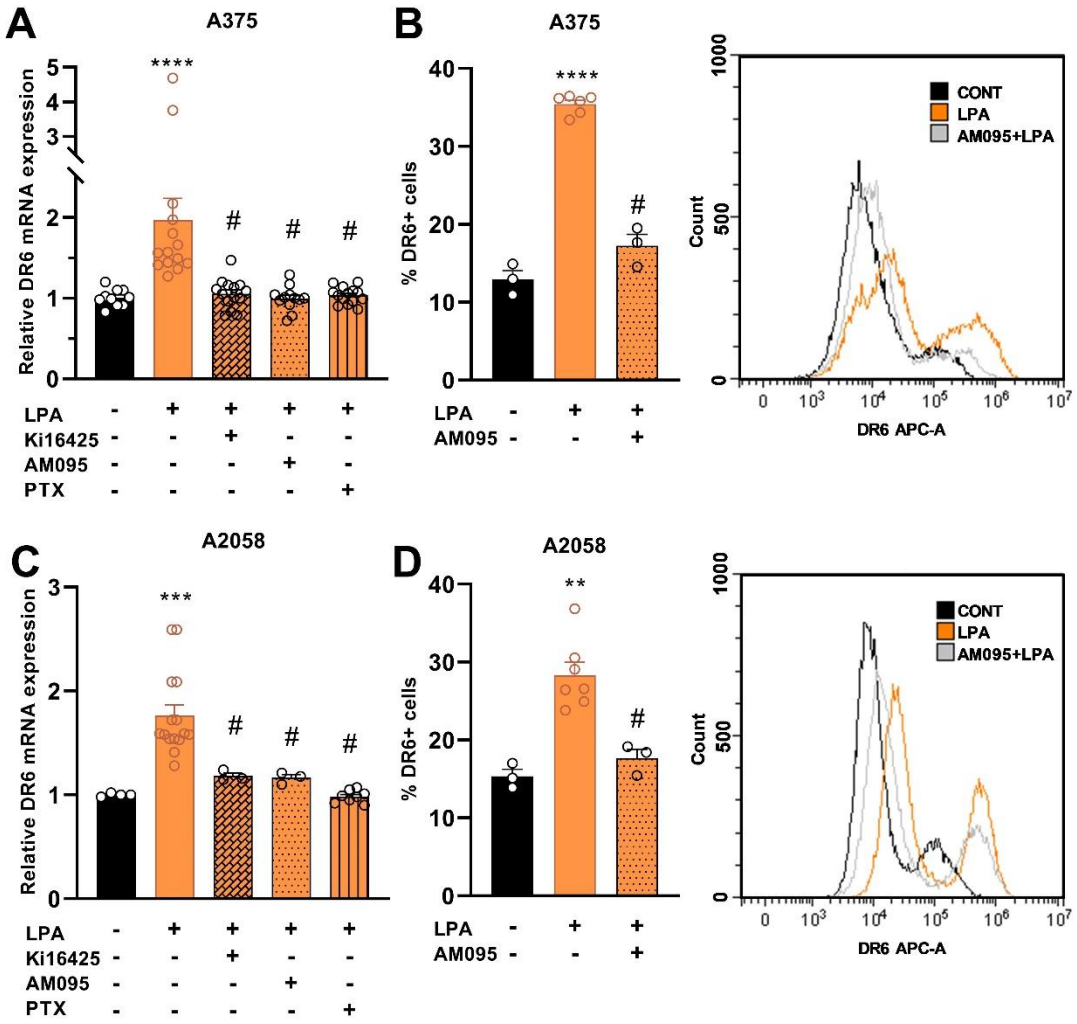

Figure 8. Melanoma cells were treated with 10 µM LPA for 1 h in the presence or absence of 10 µM Ki16425 or 10 µM AM095. The pretreatment with PTX (100 ng/mL) was administered 16 h prior to the LPA treatment. Gene expression was evaluated by qPCR (**A, C**). The inhibitory effect of AM095 on DR6 protein expression was analyzed by flow cytometry (**B, D**). All data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's posthoc test; **p<0.01, ***p<0.001, ****p<0.0001 vs. control; #p<0.001 vs. LPA alone. Figure adapted from the author's original publication (121).

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 8A and C)** (121).

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 9A)**. 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 (i.e. in the absence of LPA) **(Figure 9B)**. 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 (121).

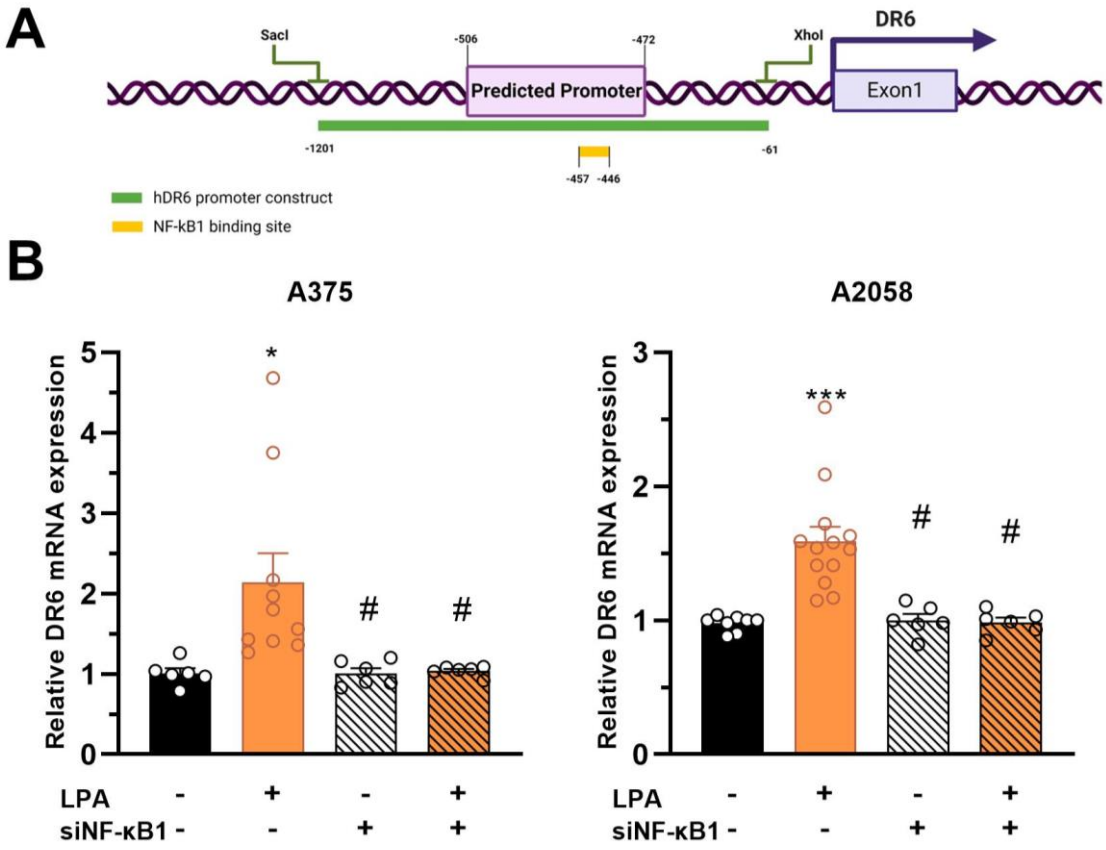

Figure 9. A. The putative promoter region of human DR6 was identified using the Berkley NNPP program; and the ALGGEN-PROMO software was used to identify the transcription factor binding sites. **B.** NF-κB1 was silenced using specific siRNA to assess its role in the LPA-mediated induction of DR6. All data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's posthoc test; *p<0.05, ***p<0.001 vs. control; #p<0.001 vs. LPA alone. Figure adapted from the author's original publication (121).

DOI:10.14753/SE.2025.3109

4.3 Regulation of IL-10 production by LPA in melanoma

As IL-10 has been reported to play a crucial role in melanoma progression (122), we investigated whether LPA could regulate IL-10 expression. We found that 10 µM LPA increased IL-10 transcript with a similar time course in A2058 and A375 melanoma cells, resulting in maximal mRNA expression at 3h **(Figure 10 A and C)**. Using AM095 and PTX, it is shown that LPA-induced upregulation of IL-10 is mediated via the LPAR1-G_i pathway **(Figure 10B and D)** (121).

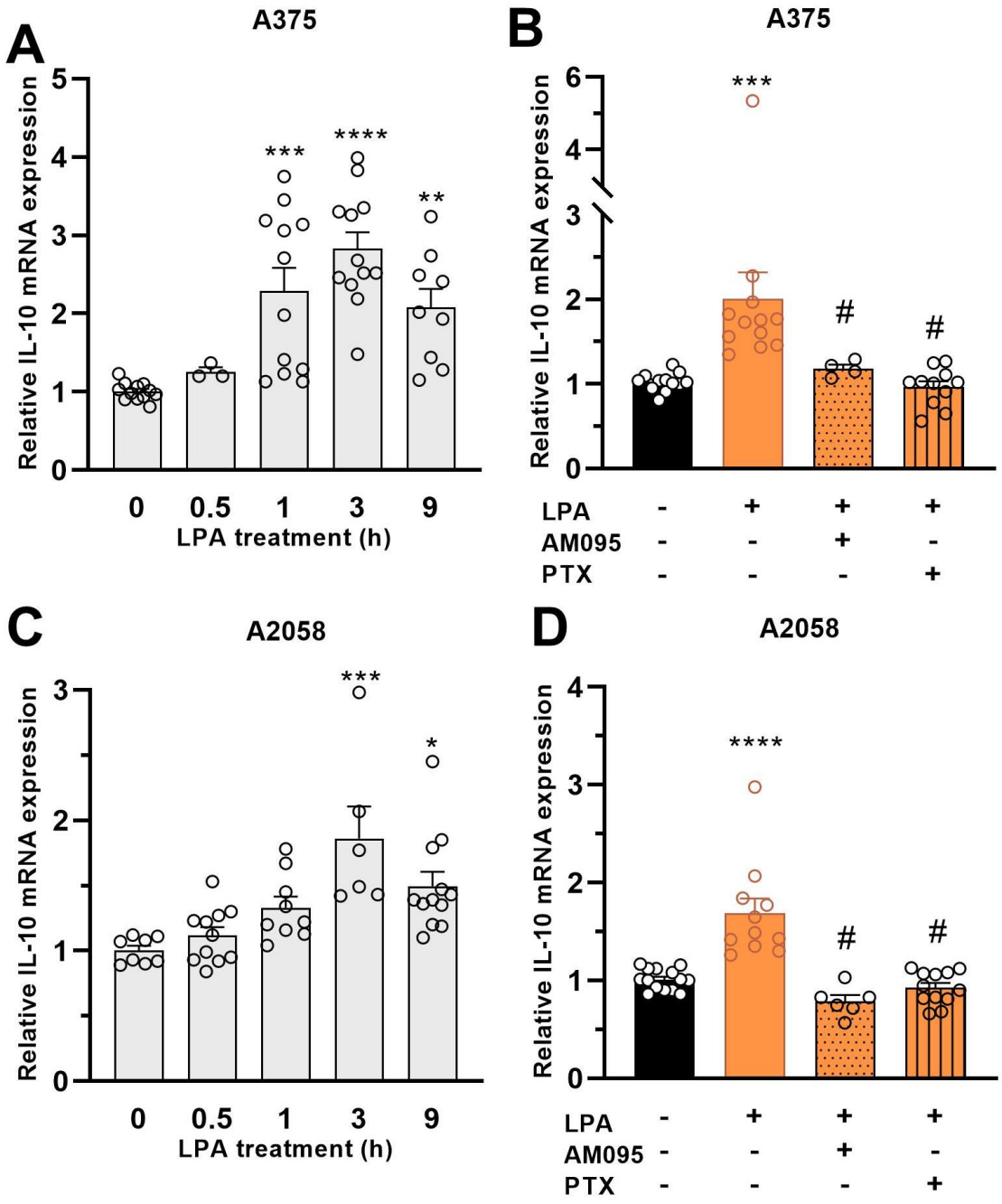

Figure 10. Melanoma cells were treated with 10 µM LPA. Time-dependent induction of IL-10 expression was examined by qPCR **(A, C)**. AM095 and PTX were used to investigate the involvement of the G_i-coupled LPAR1 in LPA-induced IL-10 expression **(B, D)**. All data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's posthoc test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001vs control, $^{\#}p$<0.001 vs. LPA alone. Figure adapted from the author's original publication (121).

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 11)**. These results indicate that DR6 is responsible for mediating LPA-induced IL-10 expression (121).

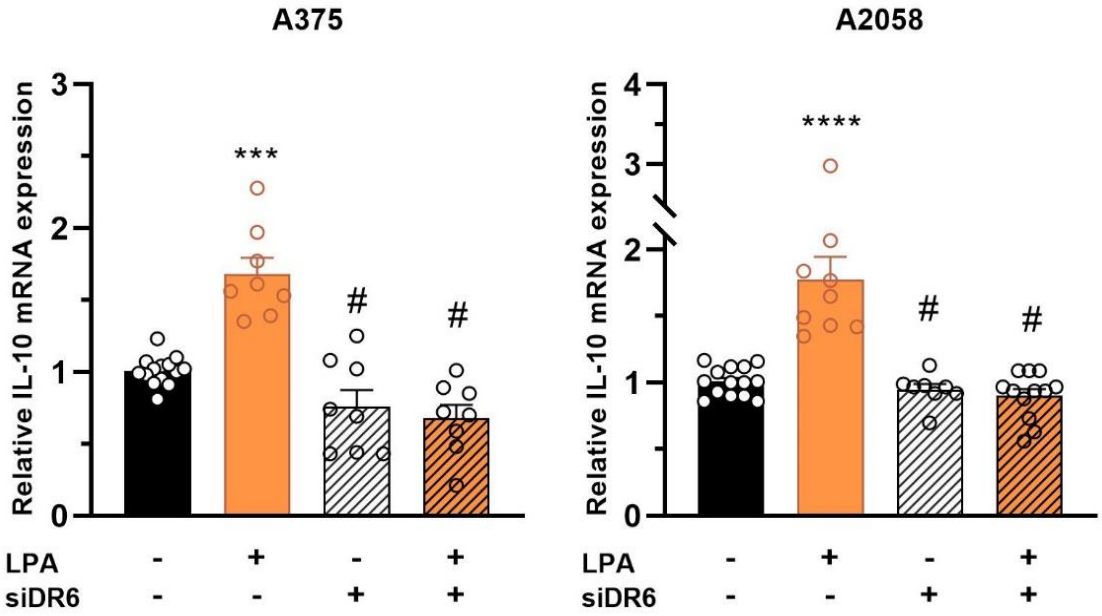

Figure 11. IL-10 mRNA expression of siNC or siDR6-transfected A375 and A2058 melanoma cells in the presence or absence of 10 µM LPA or its vehicle for 3 h. All data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's posthoc test; ***p<0.001, ****p<0.0001vs control, #p<0.0001 vs. LPA alone. Figure adapted from the author's original publication (121).

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 12)**. These effects were abolished completely by pharmacological inhibition of LPAR1 or silencing of the DR6 gene **(Figure 12)**, providing evidence for the involvement of the LPA-LPAR1-DR6 axis in increasing IL-10 secretion in melanoma (121).

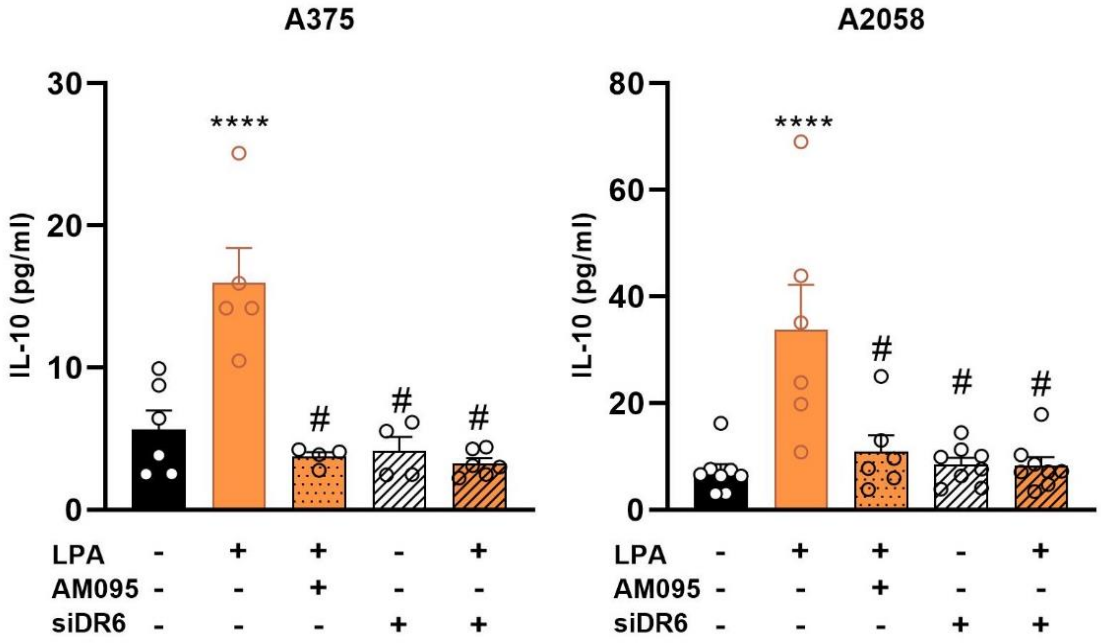

Figure 12. siNC or siDR6-transfected A375 and A2058 melanoma cells were treated with 10 μM LPA or its vehicle for 12 h in the presence or absence of AM095. The level of IL-10 was determined in the cell supernatants. All data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's posthoc test; ****p<0.0001vs control, #p<0.0008 vs. LPA alone. Figure adapted from the author's original publication (121).

4.4 Correlation of LPAR1-DR6 and IL-10 expression in melanoma patient samples

Databases of patients with melanoma were analyzed using the Spearman rank correlation to verify the significance of the LPAR1−NF-κB1−DR6−IL-10 signaling cascade *in vivo*. The correlations between the gene expression levels of LPAR1, NF-κB1, DR6, and IL-10 are depicted in **Figure 13**. Based on 435 melanoma samples, LPAR1 expression strongly correlates with NF-κB1 (Spearman's r=0.23, $p=9.8\times10^{-7}$), 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 13**). 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 13**). These results are consistent with our *in vitro* findings on the LPAR1−DR6−IL-10 signaling cascade in human melanoma (121).

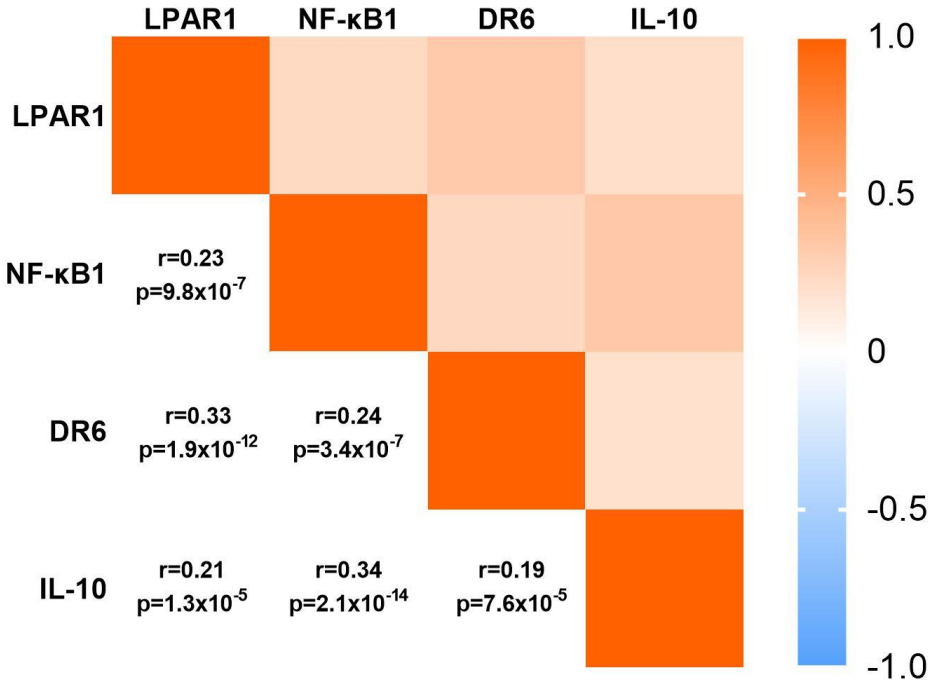

Figure 13. Heatmap representing the correlation between the expression of LPAR1, NF-κB1, DR6 and IL-10 in melanoma tumor samples. *n*= 435. Statistical analysis was performed using Spearman rank correlation. Figure adapted from the author's original publication (121).

4.5 Effect of LPA on HLA-DR expression in melanoma

Next, we investigated whether the LPA-LPAR1-DR6 axis and subsequent IL-10 release affect HLA-DR expression in human melanoma. 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 IgG1 kappa isotype control. Treatment with LPA alone resulted in a marked downregulation of HLA-DR in both A375 and A2058 melanoma cells **(Figure 14)**, which disappeared after pharmacological inhibition of LPAR1 by AM095 or silencing DR6 expression by siDR6 (121).

DOI:10.14753/SE.2025.3109

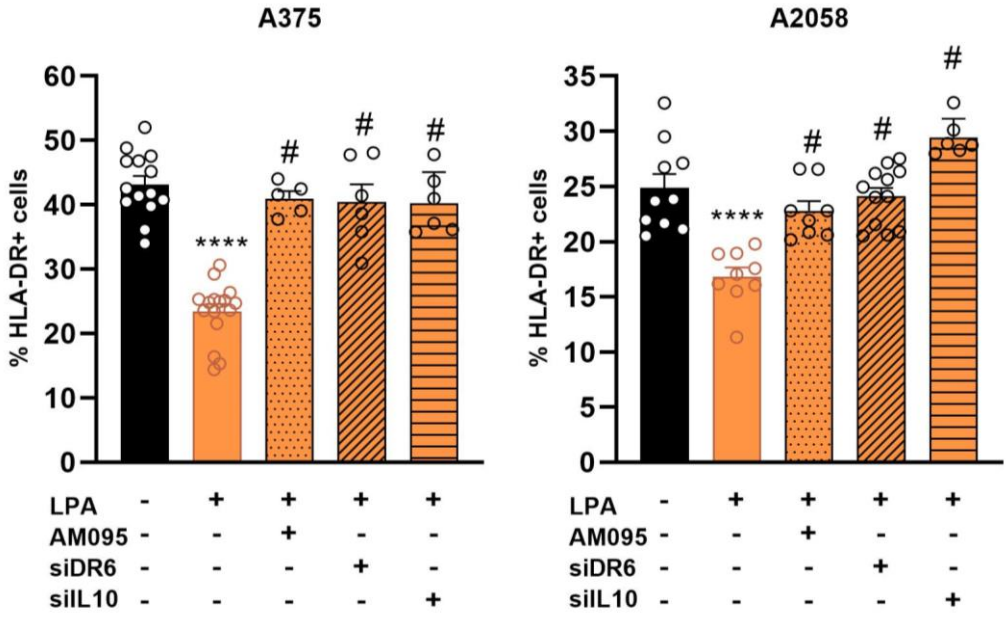

Figure 14. A375 and A2058 cells were treated with 10 μM LPA for 22 h and HLA-DR expression was measured by flow cytometry. The signaling pathway was examined using AM095, siDR6 or siIL10. All data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's posthoc test; ****p<0.0001 vs. control, #p<0.0004 vs. LPA. Figure adapted from the author's original publication (121).

More importantly, silencing IL-10 expression by siRNA **(Figure 14)** or blocking its effect by neutralizing antibody **(Figure 15)** completely abolished the effect of LPA on HLA-DR expression (121).

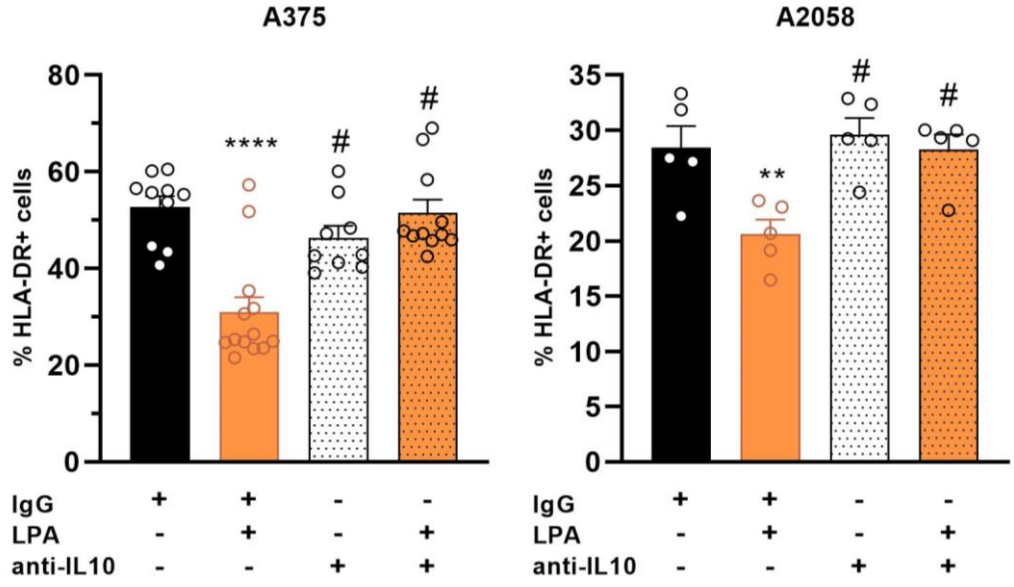

Figure 15. A375 and A2058 cells were treated with 10 μM LPA for 22 h and HLA-DR expression was measured by flow cytometry. IL-10 was neutralized by an anti-IL10 monoclonal antibody and IgG1 kappa was used as an isotype control. All data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and Dunnett's posthoc test, ***p*<0.01, *****p*<0.0001 vs. control, #*p*<0.01 vs. LPA. Figure adapted from the author's original publication (121).

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

4.6 The efficiency of anti-PD-1 therapy related to LPAR1 in melanoma patients

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 (AUC=0.574, $p=1.4\times10^{-2}$, strongest cutoff=132), confirming the marked role of LPAR1 in melanoma progression **(Figure 16)**. 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 (121).

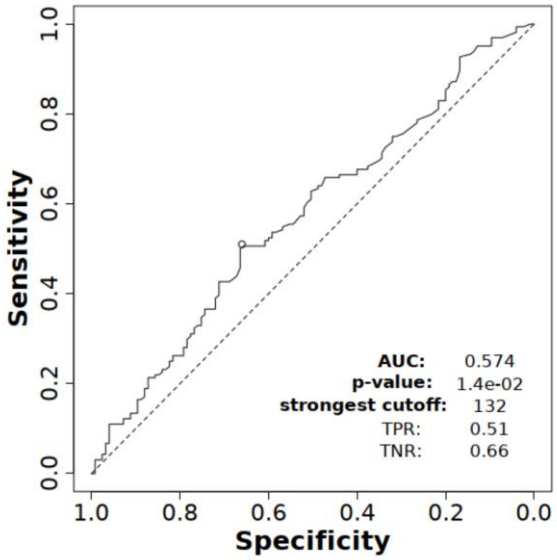

Figure 16. ROC plots of LPAR1 expression of melanoma samples predicting resistance in anti-PD-1 treatment.

DOI:10.14753/SE.2025.3109

5. Discussion

Melanoma, the most aggressive and deadly form of skin cancer, severely impacts life expectancy when it has progressed to advanced stages (87). Discovering the potency of re-invigorating the immune system revolutionized the outcomes of this malignancy. Despite the promising potential of immune checkpoint blockade for treating melanoma, about 50% of patients do not respond favorably to these therapies (87). Human A375 and A2058 melanoma cell lines were used as a preclinical model of low or highly metastatic melanoma, respectively, to investigate the molecular mechanisms by which melanoma can evade the anti-tumor immune response (121).

The evidence of LPA in tumor development and metastasis is well-established in experimental and clinical studies (123). The level of LPA in the plasma is in nanomolar concentration, but under certain pathological circumstances like malignancies, it can be increased several-fold (124). The abundant expression of autotaxin, the enzyme responsible for the biosynthesis of LPA, influences tumor development and antitumor immunity not only in melanoma (10,125–128). The autotaxin-derived LPA on tumor-infiltrating lymphocytes and its role in the immune escape of melanoma has been well-documented (10). In the current study, an LPA-mediated signaling pathway in melanoma immune-escape was examined.

Our findings demonstrate that LPA regulates DR6 expression by activating LPAR1. The altered expression of LPAR1 is linked to various aspects of carcinogenesis and therapy resistance in melanoma (14, 60,129,130). Ki16425 and AM095 were selected in our study as LPA receptor antagonists because of their well-documented pharmacological properties validated in cellular systems and preclinical animal models (5,118,119). Ki16425 is one of the most frequently used selective LPA receptor antagonists. Based on the structure of Ki16425, AM095, a biphenyl-substituted isoxazole analog, was designed and synthesized as a selective antagonist of the LPAR1 receptor (118). Therapeutic potency of LPAR1 antagonism by Ki16425 or AM095 has been reported in preclinical models of rheumatoid arthritis, hydrocephalus, dermal, pulmonary, and renal fibrosis, as well as in different tumor types (5, 15,131,132). The role of LPAR1 in LPA-induced cancer invasion and oncogenesis is well-documented (6). Elevated LPAR1 expression in many primary tumors is associated with increased cell proliferation

and poor prognosis (130). Furthermore, LPAR1 is a key regulator of melanoma invasion, metastasis, and therapy resistance mainly via G_i, G_q coupling (13, 129,133). Our experiments using PTX confirm the involvement of the LPAR1 coupled G_i protein in this regulatory process (134). Notably, this study is the first to explore how LPA and LPAR1 influence HLA-DR expression and the efficacy of anti-PD-1 therapy.

DR6 has a complex role in the progression of malignancies (135). While increased DR6 expression has been observed in various tumor types, including melanoma, its precise function in tumor biology has been unclear. Notably, DR6 upregulation is related to therapy resistance in melanoma (136).

Our study indicates that DR6 functions as an immediate-early gene responsive to LPA, as an increased DR6 promoter activity and mRNA expression were observed after 30 minutes (121). Analysis of the putative promoter sequence of DR6 revealed conserved binding sites for NF-κB1 (p50). Silencing NF-κB1 inhibited LPA-induced DR6 expression without affecting its basal expression level, confirming its role in DR6 upregulation (121). The multifaceted role of NF-κB in tumor progression is well established. NF-κB transcription factors are key regulators of cell survival, and aberrant NF-κB signaling has been implicated in the pathogenesis of most human malignancies, including melanoma (137,138). Members of the NF-κB family, especially p50 and p65, are overexpressed in melanoma cells compared to non-transformed melanocytes, emphasizing their contribution to cancer progression (139,140). The NF-κB-regulated cytokines and chemokines, when transcriptionally activated, are thought to enhance melanoma progression through autocrine and paracrine signaling (138). LPA is a known activator of NF-κB expression (141,142). However, not all cell types respond uniformly to a given stimulus, either because they lack the cognate receptor or because they lack the required signal transduction molecules (142). Although this study focused on DR6 and NF-κB in melanoma cells, their roles in immune cells are likely equally important. For instance, tumor-derived DR6 influences dendritic cell development and T-cell activation (143). NF-κB may play an even more significant role in tumor progression by regulating the release of various chemokines and cytokines from melanoma cells and the effects of these mediators are considered to promote tumor progression as they maintain a high constitutive activation of NF-κB, which switches from pro-apoptotic to anti-apoptotic functions in melanoma (138). Moreover, it is likely to be involved in the transcriptional

regulation of IL-10 expression and potentially mediating IL-10 effects. Interestingly, DR6 reportedly induces the activation of NF-κB (144), and Cao et al. identified NF-κB1, as a crucial factor in the transcription of IL-10 in macrophages (145). It is reasonable to hypothesize that NF-κB induces the expression of both DR6 and IL-10 in our system. However, due to methodological constraints, we could not clarify this in the current study.

Altered IL-10 levels can significantly contribute to carcinogenesis and tumor progression. Elevated IL-10 promotes tumor growth by activating STAT3, inhibiting apoptosis, and allowing immune evasion through downregulation of HLA class I molecules, suppression of DC function, recruitment of Treg cells, inhibition of NK cell activity, and impaired activation of CD4+ Th1 and CD8+ cytotoxic T cells (146–148). High IL-10 expression in primary tumor cells and tumor-associated macrophages is linked to cancer progression and metastasis, and elevated serum IL-10 levels in cancer patients often correlate with poor prognosis (147,149–153). Clinical studies have demonstrated a correlation between elevated IL-10 expression and melanoma progression (154–157), and Sato et al. identified transformed melanocytes as a main source of IL-10 production in melanoma metastases (157).

While LPA is recognized for its role in cytokine release, its involvement in regulating IL-10 expression by tumor cells has not been previously documented. In LPS-stimulated human dendritic cells and macrophages, LPA enhances IL-10 release, which subsequently suppresses TNF-α production (158). Additionally, DR6 serves as an immunosuppressive factor, hindering the proliferation and migration of B and T cells, thus contributing to tumor survival and progression (135). Although the upregulation of IL-10 within the tumor microenvironment is frequently reported (94,159), this study is pioneering in demonstrating that LPA induces IL-10 production in melanoma cells and highlights the LPAR1-dependent upregulation of DR6 as a key mechanism in the process.

IL-10 can impede crucial stages of immune recognition by reducing the expression of HLA class I and II antigens and the intercellular adhesion molecule-1 (ICAM-1) on the surface of melanoma cells (160). The success of immunotherapy relies on T cells being able to identify these cell surface antigens. HLA-DR, an MHC class II antigen, is constitutively expressed in antigen-presenting cells but can also be induced in other tissues, including tumor cells (161). The primary function of HLA-DR is to present antigens to CD4+ T cells, which in turn support the activation of CD8+ T cells and the

generation of memory T cells (162). Additionally, tumor-specific HLA-DR expression is linked to favorable outcomes in cancer patients (87, 92,162), and therefore it is considered a prognostic marker (91,163,164).

While HLA-DR expression does not appear to be a prognostic factor by itself in melanoma, it has been demonstrated to exert a considerable influence on the efficacy of ICI therapy (87, 89,165–168). Recent retrospective clinical studies have demonstrated a correlation between HLA-DR positivity in tumor cells and a higher response rate to ICI immunotherapy in patients with advanced melanoma (168–170). Tumors exhibiting elevated HLA-DR expression demonstrated markedly superior progression-free and overall survival rates compared to those with HLA-DR negativity (defined as tumors with less than 5% HLA-DR+ melanoma cells) (87). Our findings demonstrate that LPA markedly diminishes HLA-DR expression in human melanoma cells, thereby elucidating a previously unidentified mechanism through which LPA facilitates immune evasion. This effect is primarily mediated by the upregulation of DR6 and the subsequent release of IL-10. However, the direct impact of DR6 on HLA-DR cannot be entirely ruled out based on the available data. Furthermore, we observed a negative correlation between LPAR1 expression and the efficacy of anti-PD-1 therapy, indicating that LPA-induced downregulation of HLA-DR may contribute to therapy resistance. Notably, a recent study by Kovács et al. reported significantly higher LPAR1 expression in non-responders to ICI therapy in multiple tumor types (116). Emphasizing the role of LPA in anti-PD-1 therapy efficacy, Konen et al. identified upregulated levels of ATX and LPA in anti-PD-1 resistant non-small cell lung cancer and a negative correlation with the number of infiltrating CD8+ T cells (171), indicating that increased LPA levels negatively affect the response to ICI.

In conclusion, the findings of this study demonstrate that LPA enhances DR6 expression via a G_i-coupled LPAR1- and NF-κB-dependent mechanism. Furthermore, LPA markedly regulates IL-10 gene transcription and protein release via DR6, resulting in diminished HLA-DR expression in melanoma cells **(Figure 17)**. Given that IL-10 plays a multifaceted role in tumor immune evasion and neutralizing IL-10 has been proposed as a novel anti-tumor therapy (172,173), the downregulation of HLA-DR via LPA-induced IL-10 production may represent a critical pathway in the progression, metastasis, and immune evasion of melanoma.

DOI:10.14753/SE.2025.3109

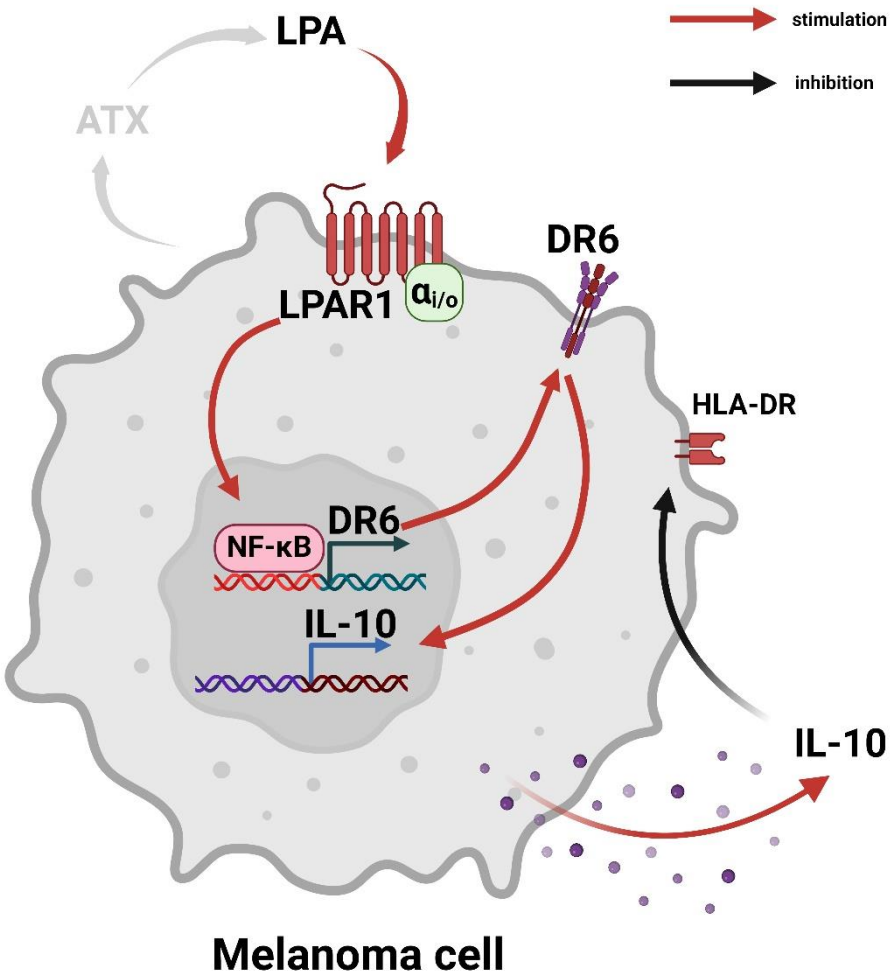

Figure 17. LPA, via its G_i-coupled LPAR1 receptor, activates NF-κB1-mediated DR6 expression, inducing the transcription and secretion of IL-10, which in turn leads to the downregulation of HLA-DR antigen in human melanoma cells. (Created with BioRender.com). Figure adapted from the author's original publication (121).

DOI:10.14753/SE.2025.3109

6. 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 G_i protein inhibitor, abrogates the effect of LPA, indicating a G_i 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-G_i 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.

DOI:10.14753/SE.2025.3109

7. Summary

Immunotherapy has revolutionized the treatment of melanoma, providing improved progression-free survival and enhanced quality of life for patients. However, despite the advances, a significant number of patients exhibit resistance to immunotherapies such as anti-PD-1, emphasizing the need to understand the underlying mechanisms of resistance and identify potential targets to enhance therapeutic outcomes. This study focuses on the role of lysophosphatidic acid (LPA) in modulating the expression of death receptor 6 (DR6) and its implications for melanoma progression and response to immunotherapy.

Recent findings have demonstrated that elevated expression of human leukocyte antigen-DR (HLA-DR) in tumors is associated with better prognosis and improved response to immune checkpoint inhibitors (ICIs). In this study, we identified LPA as a negative regulator of HLA-DR expression in melanoma cells via induction of DR6 expression. DR6, which is inducibly expressed in tumor cells, regulates various cellular functions, including cytokine release.

Our results show that LPA activates the G_i-coupled LPA receptor subtype 1 (LPAR1) signaling pathway, leading to NF-κB-mediated transcriptional upregulation of DR6 in human melanoma cells. Subsequently, LPA, through DR6, increases the expression and release of interleukin 10 (IL-10), which in turn reduces HLA-DR expression. Moreover, we found a statistically significant correlation between the expression levels of LPAR1, NF-κB, DR6, and IL-10 in human melanoma tissues, as well as our data revealed an association between increased LPAR1 expression and reduced effectiveness of anti-PD-1 immunotherapy.

These findings support the hypothesis that the LPAR1-DR6-IL-10 autocrine loop may constitute a novel mechanism by which tumor cells evade immunosurveillance, via decreasing HLA-DR expression. A better understanding of this pathway could provide new insights into overcoming resistance to immunotherapy in melanoma patients.

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9. Bibliography of the candidate's publications

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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**

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DOI:10.14753/SE.2025.3109

10. Acknowledgments

I would like to express my deepest gratitude to my supervisor, Professor Zoltán Benyó, for his unwavering support, professional advice, and guidance throughout my PhD. I would also like to express my special gratitude to Professor Gábor Tigyi for his invaluable knowledge and advice, as well as for the opportunity to learn from his research team in Memphis. I appreciate the efforts of all my co-authors. Lastly, I would like to thank my loved ones for their continuous support and encouragement throughout my life.