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Development of a PD-L1 Overexpression System in HEK293T Cells: A Platform for Studying Immune Evasion Mechanisms

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Abstract:

Programmed death-ligand 1 (PD-L1) is a key immune checkpoint protein that facilitates tumor immune evasion by interacting with PD-1 receptors on T cells, making the PD-1/PD-L1 axis a critical target in cancer immunotherapy. However, resistance to these therapies remains a challenge, necessitating further exploration of PD-L1's functions and regulatory mechanisms. In this study, we amplified the PD-L1 coding sequence (CDS) from human cDNA, cloned it into a pcDNA3.1 vector, and transfected it into HEK293T cells to establish a robust in vitro PD-L1 overexpression system. Quantitative RT-qPCR demonstrated a 300-400 fold increase in PD-L1 mRNA expression compared to controls. These results confirm the successful construction of the PD-L1 overexpression plasmid and validate its application as a tool for studying PD-L1's role in immune modulation and its potential implications in overcoming resistance to immunotherapy. This system provides a valuable platform for future investigations into PD-L1-mediated mechanisms of immune evasion and cancer progression.

Keywords: PD-L1, immune checkpoint, cancer immunotherapy, tumor microenvironment, overexpression system, HEK293T cells

1. Introduction

Although TME varies depending on the type of tumor, signature features of TME include immune cells, stromal cells, blood vessels, and the extracellular matrix. TME is an important player in promoting cancer progression. Cancer cells and TME components form a reciprocal relationship. Cancer cells recruit immune cells in TME. Causing immune cells to change from anti-tumor to pro-tumor. TME also promotes the growth of blood vessels to combat hypoxia and nutrient deficiency in the environment (Anderson and Simon, 2020). The characteristics of cancer include uncontrolled cell growth and the ability to invade tissues and metastasize to other places in the human body. The reason why TME is a crucial key for cancer is it contain cell that can kill or support tumors (Whiteside, 2008).

1.1 Current Research Landscape

Nowadays the research landscape of cancer research is focused on the tumor microenvironment (TME). TME comprises various cell types, such as immune cells, all interacting with tumor cells. TME can influence tumor growth depending on the environment. For example, a tumor can create a TME that is beneficial to the tumor by recruiting seemingly normal cells. For example, in TME, regulatory T cells suppress anti-tumor responses by secreting IL-2 and other ways to promote tumor development. Regulatory T cells also secrete growth factors to support the survival of cancer cells. In the later stages of tumor development, neutrophils in TME promote blood vessel growth by modifying the extracellular matrix and releasing VEGP. Thus promoting tumor progression and growth.(Anderson and Simon, 2020). Recent studies had highlighted the importance of targeting TME to improve the efficacy of cancer treatments, including immunotherapy (Hanahan and Weinberg, 2011).

1.2 PD-L1 and Its Role in Cancer Immunotherapy

Programmed death-ligand 1 (PD-L1, also known as CD279 and B7-H1). Belonging to the B7 series, it is a 33 kDa type 1 transmembrane glycoprotein containing 290 amino acids with Ig- and IgC domains in extracellular regions (Han, Liu and Li, 2020). The physiological function of the PD-1/PD-L1 pathway is to control the ongoing inflammatory response and prevent the spread of inflammation (Ghosh,Luong and Sun, 2021). However, it induces and maintains immune tolerance in the tumor microenvironment. (Han, Liu and Li, 2020) Programmed death-ligand 1 (PD-L1) is an important immune checkpoint protein for immune escape. PD-L1 can be expressed in the tumor cells and other inhibit T cell activity and allow tumor cells to have immune escape. The treaty of using PD-1 cancer cells again (Chen and Mellman, 2013).

1.3 T Cells and Neutrophils in the Tumor Microenvironment

T Cells are very important in tumor immunity. For example, Cytotoxic T cells can kill tumor cells and helper T cells can assist other immune cells to improve their functions. Immune checkpoints suppress the immune system, maintaining tolerance and reducing immune system damage to tissues during normal physiological functions. However, in the pathological function, immune checkpoints such as PD-L1 can bind with the receptor on the T Cells and inhibit its activity (Pardoll, 2012).

Neutrophils are traditionally known for their role in inflammation and bacterial infections. Recently, it has been discovered that it has dual effects on cancer cells in TME. Tumor-associated neutrophils (TANs) will contribute to tumor progression by promoting angiogenesis, inhibiting anti-tumor immunity, and promoting tumor metastasis. In contrast, certain subpopulations of neutrophils can exert anti-tumor effects by directly killing tumor cells or enhancing the activity of other immune cells (Fridlender et al., 2009).

2. Methods

2.1 Cell culture

1) Cell origin

HEK293 human embryonic kidney cell

2) Complete medium configuration

The formulation was RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin and streptomycin.

Table 1. HEK293T cell culture medium formulation

Type	Dosage
FBS	5 mL
penicillin and streptomycin	0.5 mL
RPMI 1640 medium	44.5 mL

2.2 Plasmid construction-Construction of high expressed plasmid

1) Target fragment extraction

a. Target fragment:

ATGAGGATATTTGCTGTCTTTATATTCATGACCTACTGGCATTTGCTGAACGCATTTACTGTCACGGTTCCCAAGGACCTATATGTGGTAGAGTATGGTAGCAATATGACAATTGAATGCAAATTCCCAGTAGAAAAACAATTAGACCTGGCTGCACTAATTGTCTATTGGGAAATGGAGGATAAGAACATTATTCAATTTGTGCATGGAGAGGAAGACCTGAAGGTTCAGCATAGTAGCTACAGACAGAGGGCCCGGCTGTTGAAGGACCAGCTCTCCCTGGGAAATGCTGCACTTCAGATCACAGATGTGAAATTGCAGGATGCAGGGGTGTACCGCTGCATGATCAGCTATGGTGGTGCCGACTACAAGCGAATTACTGTGAAAGTCAATGCCCCATACAACAAAATCAACCAAAGAATTTTGGTTGTGGATCCAGTCACCTCTGAACATGAACTGACATGTCAGGCTGAGGGCTACCCCAAGGCCGAAGTCATCTGGACAAGCAGTGACCATCAAGTCCTGAGTGGTAAGACCACCACCACCAATTCCAAGAGAGAGGAGAAGCTTTTCAATGTGACCAGCACACTGAGAATCAACACAACAACTAATGAGATTTTCTACTGCACTTTTAGGAGATTAGATCCTGAGGAAAACCATACAGCTGAATTGGTCATCCCAGAACTACCTCTGGCACATCCTCCAAATGAAAGGACTCACTTGGTAATTCTGGGAGCCATCTTATTATGCCTTGGTGTAGCACTGACATTCATCTTCCGTTTAAGAAAAGGGAGAATGATGGATGTGAAAAAATGTGGCATCCAAGATACAAACTCAAAGAAGCAAAGTGATACACATTTGGAGGAGACGTAA

b. Amplification system (20μg)

Table 2. Target gene PCR amplification system

Reagent	Dosage
Forward primer (10μM)	0.5 μL
Reverse primer (10μM)	0.5 μL
cDNA	1 μL
Compound 2x buffer	10 μL
ddH_2O	8 μL

c. Amplification program

Table 3. Target gene PCR amplification program

Step	Temperature	Time
Predegeneration	95℃	5 min
Degeneration	95℃	30 s
Annealing	58℃	30 s
Extension	72℃	1 min
	Repeat steps 2-4 for 34 cycles	
	72℃	10 min
	12℃	Preserve

d. AGE (agarose gel electrophoresis) (1.5%/20mL)
Weigh 0.3g agarose powder and put it into a conical bottle containing 20mL 1×TAE. Heat it in a microwave oven for 1 minute until the agarose powder is completely dissolved and the solution becomes clear. Wait until the solution cools to 60℃, and add 3 μL EB. To avoid bubbles and homogenize the solution, gently shake the solution. Assemble the gel equipment and add the solution. Leave it stand horizontally at room temperature for 30 minutes until the gel sets. Add the PCR product into the gel hole and the marker at the appropriate position. Electrophoresis was performed at a constant voltage of 110V for 22 minutes. The gel is removed and placed in a gel imager to be imaged with ultraviolet light. Cut the target strip with a clean blade and collect it into a clean 1.5mL EP tube.
e. Gel extraction
The PCR products were recovered according to the instructions of the glue recovery kit. The product concentration was measured with the Nanodrop 2000 instrument and temporarily stored in the -20℃ refrigerator.
2) Vector double digestion linearization
a. plasmid: pcDNA3.1
b. Enzyme digestion system (20μL):

Table 4. Linearized system of plasmid vectors

Reagent	Dosage
EcoR I	0.5 μL
Xba I	0.5 μL
10×Cutsmart buffer	0.5 μL
plasmid	2 μg
H_2O	14.3 μL
enzyme cut overnight with 37℃ water bath	

c. AGE (agarose gel electrophoresis) (0.5%/40mL)
Weigh 0.2g agarose powder and put it into a conical bottle containing 40mL 1×TAE. Heat it in a microwave oven for 1 minute until the agarose powder is completely dissolved and the solution becomes clear. Wait until the solution cools to 60℃, and add 5 μL EB. To avoid bubbles and homogenize the solution, gently shake the solution. Assemble the gel equipment and add the solution. Leave it stand horizontally at room temperature for 30 minutes until the gel sets. Add the PCR product into the gel hole and the marker at the appropriate position. Electrophoresis was performed at a constant voltage of 110V for 22 minutes. The gel is removed and placed in a gel imager to be imaged with ultraviolet light. Cut the target strip with a clean blade and collect it into a clean 1.5mL EP tube.
d. Gel extraction
The PCR products were recovered according to the instructions of the glue recovery kit. The product concentration was measured with the Nanodrop 2000 instrument and temporarily stored in the -20℃ refrigerator.
3) Connect target fragment to a linearized vector
The 0.03pmol linearized carrier and 0.06pmol target fragment were calculated and absorbed into a PCR tube, added with 5μL homologous recombinase, mixed with a pipette, centrifuged instantaneously and placed in a water bath at 50℃ for 60min.

4) Conversion

a. Preheat water bath at 42℃.

b. Escherichia coli DH5a receptor cells were removed from the -80℃ refrigerator. Place on ice to thaw slowly.

c. The homologous recombinant products were added into the thawed receptive cells, mixed and placed on ice for 30min.

d. After standing, place the EP tube in a water bath at 42℃ for 60s and then place it on ice for 3min immediately.

e. 900μL LB liquid medium (without antibiotics) was added into the EP tube on a clean bench, and the bacteria were shaken at 220rpm in a shaking table at 37℃ for 1h.

f. The LB solid plate containing ampicillin was preheated in a 37℃ incubator.

g. After taking the shaken bacteria, the EP tube was centrifuged at 3000rpm for 3min, and 900μL supernatant was sucked up and discarded on a clean workbench. The weight of the bacteria was suspended with the remaining medium, and evenly coated on the LB solid plate containing ampicillin with a sterile coating stick.

h. Put it into an incubator at 37℃ and culture it upside down for 12-16h.

2.3 Cell transfection

a. 125μL Opti-MEM and 9μL PEI were added into 1.5 mL EP tube according to the amount of transfection agent PEI (μL), which was three times the amount of plasmid transfection (μg), then mixed with pipetting gun, added 3 μg plasmid vector, mixed and left for 15 minutes at room temperature.

b. Discard the medium of untransfected cells. Add 1 mL of serum-free and antibiotic-free medium, and after the transfection plasmid was standing, the plasmid-PEI complex was absorbed and added to the cells by drops.

c. Put it in a 37℃ incubator.

d. Replace 2 mL fresh and complete medium after 6 hours. Continue to culture until 48 hours to collect cells for subsequent experiments.

2.4 Western Blot Analysis

Cells or aortas were lysed in RIPA buffer to prepare whole protein samples. Equal amounts of total protein were separated using SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies at 4°C overnight, followed by IRDye-conjugated secondary antibodies incubation at room temperature for 1 hour. The immunofluorescence signals were obtained by Odyssey infrared imaging.

3.Results

3.1 Successful Construction of Human PD-L1 Overexpression Plasmid In Vitro

We successfully amplified the human PD-L1 CDS region from the template using specific primers targeting the Homo sapiens CD274 molecule (CD274), transcript variant 1. The PCR reaction produced the desired PD-L1 CDS fragment, which was confirmed by agarose gel electrophoresis. The gel electrophoresis results demonstrated a single band of approximately 872 bp, corresponding to the expected size of the PD-L1 CDS region (Fig. 1). The band was excised from the gel, and DNA was recovered using the gel extraction method. Subsequent ligation of the purified fragment into a linearized pcDNA3.1 vector generated the PD-L1 overexpression plasmid, which was confirmed through sequencing. This successful construction of the plasmid provided the foundation for establishing an in vitro PD-L1 overexpression system.

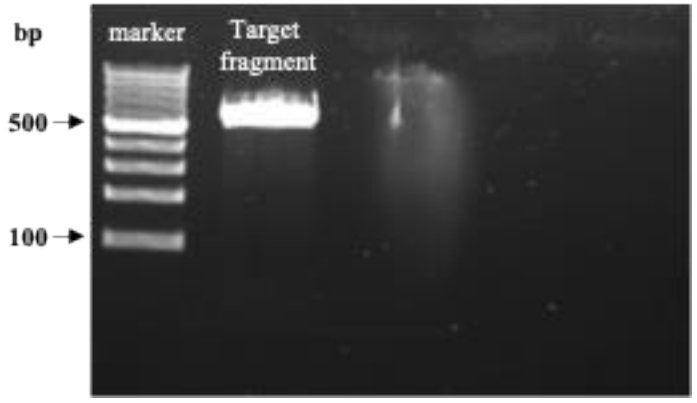

Figure 1. Results of nucleic acid electrophoresis of PD-L1 CDS region

3.2 Establishment of a PD-L1 Overexpression System in HEK293T Cells

Following the successful construction of the PD-L1 overexpression plasmid, we transfected the plasmid into HEK293T cells to create an in vitro overexpression system. HEK293T cells were seeded into 6-well plates and transfected when they reached 80% confluence using a

PEI-based transfection reagent. After 24 hours, cells were harvested, and total RNA was isolated. The RNA was reverse transcribed into cDNA, and RT-qPCR was performed to assess PD-L1 expression levels.

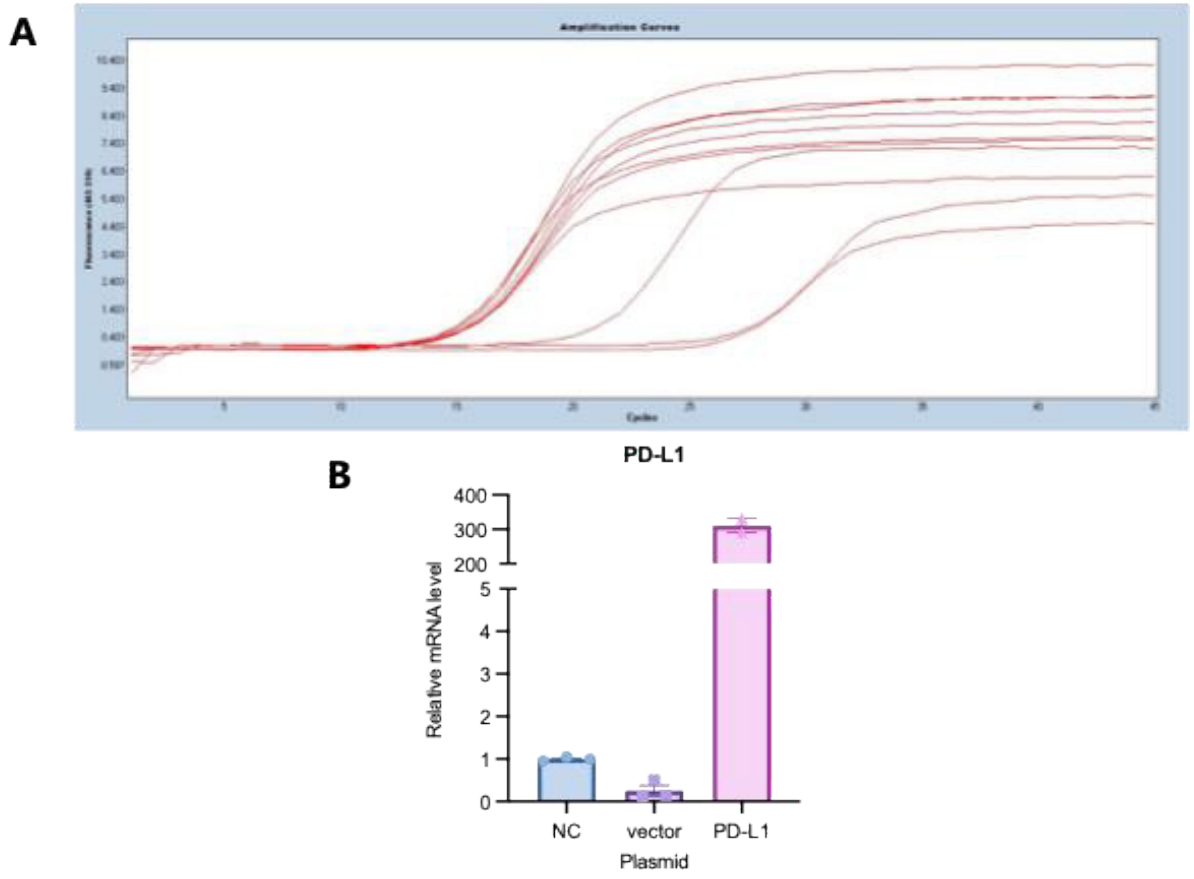

Figure 2. RT-qPCR results showed the expression level of PD-L1 in HEK293T cells

RT-qPCR analysis revealed a substantial increase in PD-L1 mRNA levels in the transfected cells compared to the negative control cells (Fig.2A). Specifically, PD-L1 mRNA expression in the overexpression group was 300-400 times higher than that of the control group (Fig. 2B). Western blot results showed that the expression level of PD-L1 protein in HEK293T cells transfected with PD-L1 overexpression plasmid was significantly higher than that in the control group (Fig.3). These results confirmed the successful establishment of a highly efficient PD-L1 overexpression system in HEK293T cells, which will be instrumental in further functional studies involving PD-L1, particularly in the context of cancer immunotherapy. The establishment of this system allows for subsequent analyses on how PD-L1 expression affects T cell activity and immune escape mechanisms within the tumor microenvironment, providing a valuable tool for future investigations.

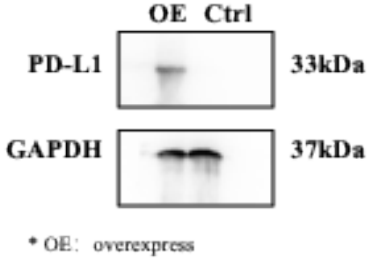

Figure 3. Western blot results showed that the expression level of PD-L1 protein in HEK293T cells

4. Discussion

The present study successfully constructed and validated a human PD-L1 overexpression plasmid and established an in vitro PD-L1 overexpression system in HEK293T cells. These accomplishments provide a foundational tool for further exploration of the role of PD-L1 in cancer immunotherapy, particularly in the context of immune evasion by tumor cells. The results demonstrate the utility of this model for studying PD-L1-mediated interactions between

tumor cells and the immune system, which is increasingly recognized as a critical determinant of tumor progression and response to therapy.

4.1 PD-L1 Overexpression System: A Platform for Investigating Tumor Immune Evasion

PD-L1 is a key component of the immune checkpoint pathway, acting as an inhibitory signal to prevent the activation of T cells, thus allowing tumor cells to evade immune surveillance. The overexpression system developed in this study allows precise control and investigation of PD-L1 function. The RT-qPCR results, which indicated a 300-400-fold increase in PD-L1 mRNA levels in transfected cells, underscore the efficiency of the system and confirm the successful manipulation of PD-L1 expression. Such overexpression models are essential for unraveling the molecular mechanisms by which PD-L1 influences tumor cell survival and immune evasion.

Recent studies have shown that high levels of PD-L1 expression on tumor cells are correlated with poor prognosis in several cancer types, including lung cancer, melanoma, and renal cell carcinoma (Oh, S. Y et al., 2021). PD-L1's interaction with PD-1 on T cells leads to the inhibition of T cell activation, proliferation, and cytokine production, ultimately promoting tumor immune escape (Gou, Q. et al., 2020). By establishing a robust PD-L1 overexpression system, this study provides a platform for further investigations into how PD-L1 modulates the tumor microenvironment (TME) and alters the balance between immune activation and suppression.

4.2 Implications for Cancer Immunotherapy

The therapeutic blockade of the PD-1/PD-L1 axis has revolutionized cancer treatment, especially for patients with tumors that express high levels of PD-L1. Anti-PD-1/PD-L1 therapies, such as nivolumab and pembrolizumab, have shown remarkable efficacy in reinvigorating T cell responses against tumors, leading to durable clinical responses in a subset of patients (Prasad, V., & Kaestner, V., 2017). However, the overall response rates remain modest, and many patients experience primary or acquired resistance to these therapies. Understanding the mechanisms underlying PD-L1-mediated immune evasion is therefore critical to improving immunotherapy outcomes.

The PD-L1 overexpression system described here can be leveraged to explore potential resistance mechanisms. One of the hypotheses under investigation is that tumors can upregulate PD-L1 expression in response to IFN-γ secretion by T cells, creating a feedback loop that protects tumor cells from immune attack (Chen, S. et al., 2019) . By manipulating the expression levels of PD-L1 in this system, it would be possible to study how changes in the tumor microenvironment, particularly the presence of inflammatory cytokines, modulate PD-L1 levels and influence therapeutic efficacy.

Furthermore, this system could be used to screen for novel therapeutic agents that target PD-L1 or its downstream signaling pathways. Beyond blocking PD-1/PD-L1 interactions, recent research has focused on disrupting the intracellular pathways activated by PD-L1, such as the PI3K/AKT and JAK/STAT pathways, which are involved in promoting tumor survival and proliferation (Yamaguchi, H. et al., 2022). The PD-L1 overexpression system provides a valuable tool for evaluating the efficacy of these emerging therapeutic strategies in a controlled and reproducible manner.

4.3 The Role of PD-L1 in the Tumor Microenvironment

The interaction between tumor cells and the immune system is a dynamic and complex process, heavily influenced by TME. The TME consists of immune cells, stromal cells, and extracellular matrix components, all of which contribute to the tumor's ability to grow, invade, and evade immune destruction. PD-L1 is not only expressed on tumor cells but also on other cells within the TME, including tumor-associated macrophages (TAMs), dendritic cells, and myeloid-derived suppressor cells (MDSCs), all of which can contribute to immune suppression.

The PD-L1 overexpression system established in this study offers a unique opportunity to investigate how PD-L1 expression in tumor cells influences the behavior of other immune cells within the TME. For instance, it could be used to explore how PD-L1 expression on tumor cells affects the recruitment and function of T cells, macrophages, and neutrophils. Neutrophils, traditionally known for their role in bacterial infections, have recently been implicated in cancer progression, with tumor-associated neutrophils (TANs) exhibiting both pro- and anti-tumor functions depending on the context (Jaillon, S. et al, 2022). Future studies using this system could provide insights into how PD-L1 modulates neutrophil behavior and its role in promoting or inhibiting tumor progression.

4.4 Limitations and Future Directions

While the PD-L1 overexpression system developed here represents a significant advancement, there are limitations to the current study that warrant further investigation. First, the study was conducted in HEK293T cells, which are human embryonic kidney cells commonly used for transfection experiments but do not naturally express PD-L1 or other key components of the TME. As such, the findings may not fully recapitulate the complexity of PD-

L1 regulation in cancer cells or within the TME. Future studies should aim to validate these findings in cancer cell lines or primary tumor cells to better understand the physiological relevance of PD-L1 overexpression in different cancer contexts.

Additionally, the functional consequences of PD-L1 overexpression in this system were not fully explored. While RT-qPCR confirmed the successful overexpression of PD-L1 at the mRNA level, future experiments should examine the protein expression of PD-L1 and assess its impact on immune cell behavior. Co-culture experiments with T cells or other immune cells would provide valuable insights into how PD-L1 overexpression affects immune evasion and tumor-immune interactions.

Finally, the application of this system to in vivo models would be an important next step. While in vitro systems provide a controlled environment for studying gene function, they cannot fully capture the complexity of tumor biology in living organisms. In vivo models, such as mouse tumor xenografts, would allow for the investigation of how PD-L1 overexpression influences tumor growth, metastasis, and response to immunotherapy in a more physiologically relevant setting.

In summary, this study successfully constructed and validated a PD-L1 overexpression plasmid and established a robust in vitro PD-L1 overexpression system in HEK293T cells. This system provides a valuable tool for investigating the role of PD-L1 in tumor immune evasion and for exploring new therapeutic strategies targeting the PD-1/PD-L1 axis. Future research should focus on validating these findings in cancer models and further elucidating the mechanisms by which PD-L1 regulates tumor-immune interactions.

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