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PD-1/PD-L1 Interaction Regulates BCL2, KI67, BAX, and CASP3, Altering Proliferation, Survival, and Apoptosis in Acute Myeloid Leukemia

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ABSTRACT

Programmed death ligand-1 (PD-L1) is a pivotal inhibitory checkpoint ligand known to induce T-cell exhaustion via interaction with the programmed death-1 (PD-1) receptor. Beyond this, PD-L1's intrinsic signaling pathways within cancer cells warrant further exploration. This study aims to elucidate the effect of PD-L1 stimulation on the proliferation, survival, and apoptosis of acute myeloid leukemia (AML) cell lines.

Two human AML cell lines, HL-60 and THP-1 were cultured and treated with phorbol 12-myristate 13-acetate (PMA) to induce PD-L1overexpression. Post-treatment PD-L1 expression was confirmed via flow cytometry. Subsequently, cell surface PD-L1 was stimulated using a recombinant PD-1, 24 hours post-PMA treatment. The expression alterations in pivotal genes including *BCL2*, *MKI67*, *BAX*, and *CASP3* were monitored using quantitative real-time polymerase chain reaction 24 and 48 hours post-treatment. Additionally, annexin-V through flow cytometry.

Findings reveal that PD-L1 stimulation augments AML cell proliferation and survival by enhancing *MKI67* and *BCL2* expressions while concurrently inhibiting cell apoptosis due to decreased *BAX* and *CASP3* expression following PD-L1 stimulation. Notably, stimulated cells expressed exhibited reduced annexin-V compared to control cells.

This study underscores that PD-L1 stimulation fosters AML cell proliferation and survival while impeding cell apoptosis. The results hold potential implications for targeting PD-L1 in AML treatment strategies.

Keywords: Acute myeloid leukemia; Apoptosis; Cell proliferation; Programmed death-ligand 1

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INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy characterized by the unregulated proliferation of bone marrow myeloid stem cells that is accompanied by heterogeneous clinical outcomes due to the complexity of its molecular and cytogenetic architecture.[1-3] Although chemotherapy is considered a standard treatment for AML, nearly half of all patients who achieve remission experience a relapse within 2 to 3 years of initial treatment.[4-6] AML blast cells overexpress various immune checkpoint receptors or ligands, which not only help evade the antileukemic immune response but can also trigger intracellular signals in cancer cells.

Programmed death ligand-1 (PD-L1 or CD274) is one of the major checkpoint ligands overexpressed on AML cells.[7] According to previous studies, PD-L1 expression is considered a prognostic factor and correlated with response to anti-PD-1 therapy in many cancer types, including AML.[8-11] Dong et al. demonstrated that the expression of PD-L1 by AML cells may directly cause the expansion of regulatory T cells (Tregs) which is considered a mechanism of immune evasion.[12]

In apoptosis, B-cell lymphoma protein-2 (Bcl-2)-associated X (Bax) protein activates a cascade of reactions by releasing cytochrome C from the mitochondria that help in successive activation of caspases and ultimately leads to cell death.[13] Bcl-2 is a central regulator of apoptosis. In many cancers, high levels of this antiapoptotic protein were shown to contribute not only to tumor initiation and progression but also to resistance to chemotherapy.[10] On the other hand, Caspase-3 is a cysteine–aspartic acid protease that is best known for its enzymatic function at the end of the intrinsic apoptotic cascade which Bax and Caspase-3 have been considered proapoptotic regulators and their expression has prognostic significance in malignancies.[14] The protein, marker of proliferation Kiel-67 (Ki-67) is also considered a cell proliferation index that is strongly associated with tumor cell proliferation and growth.[12]

PD-L1 is considered a surface ligand for PD-1. However, it has also intracellular effects in AML cells that are poorly evaluated. According to our previous study, PD-L1 also has some metabolic effects on AML cells. The impacts of PD-L1 on some metabolic and signaling pathways have partially been investigated, and these studies are mostly about solid tumors.[15-18] On the other hand, some effects of other inhibitory molecules such as Tim-3 on AML blast cells have been explored.[19] However, the direct influence of PD-L1 stimulation on apoptosis, proliferation, and survival-controlling genes, particularly in hematologic malignancies, has not been studied. In the present study, we have investigated the impact of PD-L1 stimulation on the regulation of apoptosis, proliferation, and survival of AML cells through these critical genes.

MATERIALS AND METHODS

Cell Culture and Treatment

The human AML cell lines, THP-1 and HL-60 were obtained from the Pasteur Institute of Iran (Tehran, Iran) and were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium which was fortified with 20% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) (Bio Idea, Iran). Both cell lines were plated on a 12-well plate (2×10^6 cells/well) and incubated at 37°C and 5% CO_2 overnight. Further, they were stimulated with 50 ng/mL phorbol 12-myristate 13 acetates (PMA, Sigma-Aldrich, St. Louis, MO, USA) to overexpress PD-L1 and were again incubated for 24 hours. PMA can stimulate cells as a pan-stimulator through the NF-κB pathway.[20,21] According to our previous study, PMA increases PD-L1 expression in AML cell lines.[15] To stimulate PD-L1 on cells, test group cells were supplemented with the recombinant human PD-1 (CD279)-Fc chimera (carrier-free) (BioLegend, UK) in a concentration of 80 ng/mL but control group cells were not treated.

Confirming PD-L1 Overexpression via Flow Cytometry

To confirm the expression of PD-L1 on cells before their main treatment (recombinant PD-1), flow cytometry analysis was performed 24 hours after stimulation with PMA using a FACS Calibur instrument (BD Biosciences, San Jose, USA) and antihuman CD274 (PD-L1)-PE (BioLegend, UK).

RNA Isolation and Real-time Quantitative Polymerase Chain Reaction

The total RNA content of both control and test cells was extracted 24 and 48 hours after the main treatment using an RNA extraction kit (Parstus, Mashhad, Iran)

and immediately reverse-transcribed to cDNA by using a cDNA synthesis kit (Addbio Inc., Korea) according to the manufacturer's instructions. Polymerase chain reaction (PCR) primers used in this study were designed using AlleleID 7.6 software (Premier Biosoft) and synthesized by Metabion GmbH (Planegg-Martinsried, Germany) (Supplementary Table).

Quantitative PCR was conducted using a StepOnePlus system (Applied Biosystems) with SYBR Green Real-Time PCR High ROX (Pars Tous, Iran) in the following conditions: 95°C for 15 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The expression changes of target genes that were considered markers of apoptosis, proliferation, and survival of cells, were examined using real-time PCR analysis.

Apoptosis Assay

Forty-eight hours after recombinant PD-1 treatment, cell apoptosis was assessed by staining cells with FITC-labeled annexin V and PI using Annexin V-FITC/PI Apoptosis Detection Kit (BioLegend, UK), and then the percentage of apoptotic cells was determined by flow cytometry according to the manufacturer's instructions

Statistical Analysis

Data are presented as mean±SD. We used parametric tests, t-test, and analysis of variance (ANOVA) since all data had a normal distribution. All experiments were performed in triplicates. Statistical analysis was performed using GraphPad Prism version 9.1.1. The comparison of the significance of the differences between two or more groups was analyzed with a two-way ANOVA.

RESULTS

PMA-induced PD-L1 Overexpression in AML Cell Lines

To determine the expression of PD-L1 in AML cell lines, HL-60 and THP-1 cell lines were stained with phycoerythrin. A flow cytometry assay was performed 24 hours after PMA stimulation. The results demonstrated that PD-L1 expression was increased 24 hours after PMA treatment. HL-60 and THP-1 cells had expressed PD-L1 up to 88.11% and 49.9%, respectively. (Figure-1)

PD-L1 Manipulation Increased Cell Proliferation and Survival and Decreased Apoptosis in AML Cell Lines

To investigate whether PD-L1 stimulation affects AML cell proliferation, survival, and apoptosis, the expression changes of *BCL2*, *BAX*, *CASP3*, and *KI67* were analyzed using qRT-PCR. Data analysis demonstrated that PD-L1-stimulated cells increased the expression of *BCL2* and *KI67* compared with control cells. Therefore, PD-L1 stimulation can promote AML cell survival and proliferation. (p value<0.05). On the other hand, Bax and Caspase-3 expressions decli ned following recombinant PD-1 treatment that shows PD-L1 stimulation may inhibit cell apoptosis (p value<0.05). (Figures 2, 3, 4, and 5; Table 1)

Reduced Apoptosis in PD-L1--stimulated AML Cells Compared to Controls

Flow cytometry analysis on AML cell lines HL-60 and THP-1, 72 hours after PD-L1 stimulation demonstrated that unstimulated cell groups had produced more annexin-V than PD-1–treated cells as a marker of apoptosis. Its expression in HL-60 cells was 14.34% vs 24.15% in the PD-L1–stimulated and control groups, respectively. Furthermore, in THP-1 cells, annexin-V had been expressed at 39.67% vs 64.57% in the PD-L1–stimulated and controls, respectively (Figure 6).

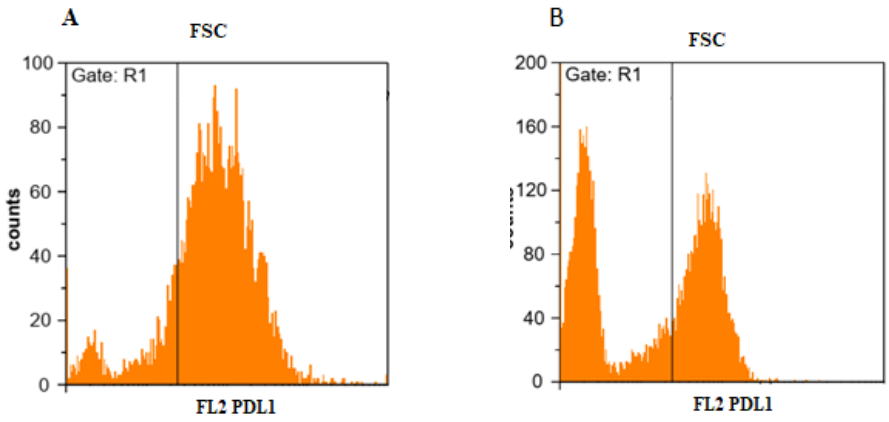

Figure 1. PD-L1 Expression 24 hours after phorbol 12-myristate 13 acetate (PMA) treatment using antihuman CD274 (PD-L1)-PE. A) Histograms of HL-60 cells expressing PD-L1 in 24 hours after PMA treatment. B) Histograms of THP-1 cells expressing PD-L1 in 24 hours after PMA treatment.

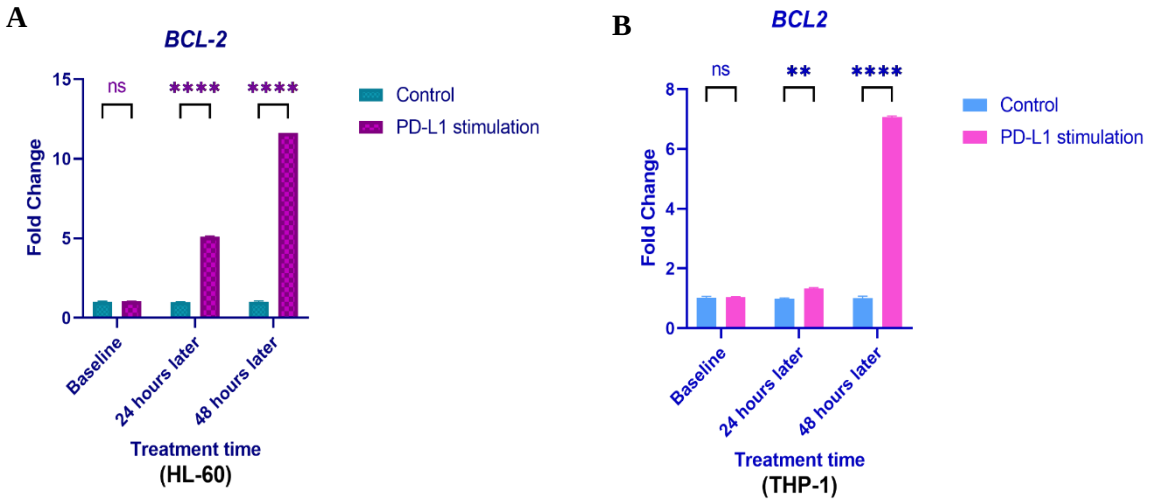

Figure 2. Expression changes of B-cell lymphoma protein-2 (*BCL2)* at baseline, 24, and 48 hours after PD-L1 stimulation in stimulated cells compared with control. Most changes occurred 48 hours after treatment. A) In the HL-60 cell line, *BCL2* expression increased after PD-L1 stimulation while no expression changes were observed in unstimulated (i.e., control) cells. B) In the THP-1 cell line, *BCL2* expression increased after PD-L1 stimulation while no expression changes were observed in unstimulated or control cells. ns: not significant and **p<0.01, ****p<0.0001

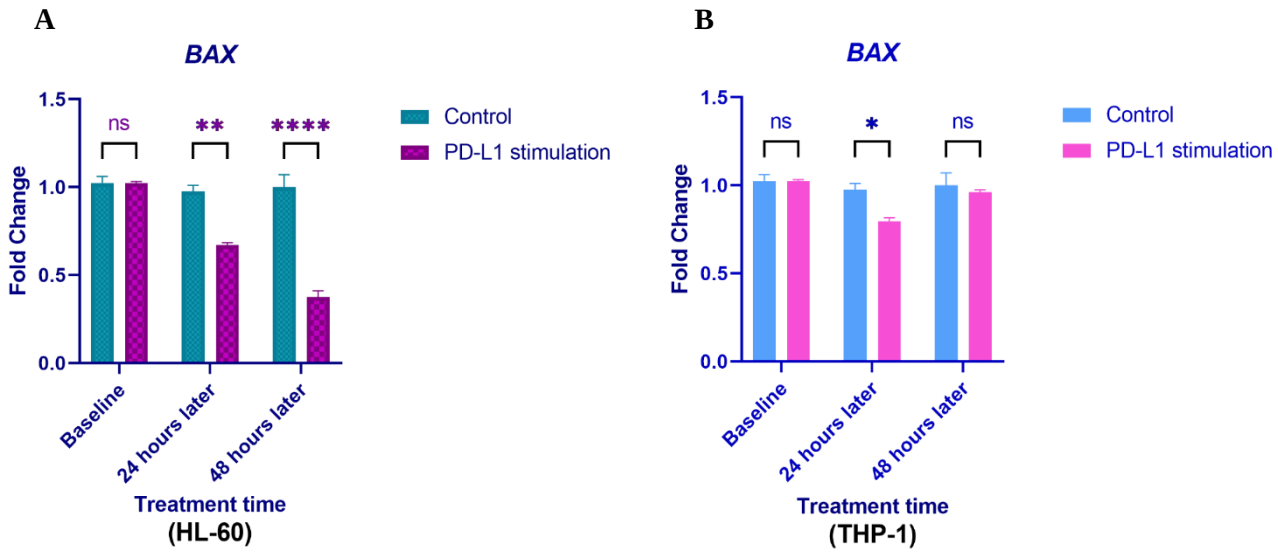

Figure 3. Expression changes of *BAX* at baseline, 24 and 48 hours after PD-L1 stimulation in stimulated cells compared with control. A) In the HL-60 cell line, *BAX* expression decreased after PD-L1 stimulation and most changes occurred 48 hours after treatment. No expression changes were found in the control (unstimulated) cell group. B) In the THP-1 cell line, *BAX* expression was decreased after PD-L1 stimulation and the expression changes were significant as early as 24 hours after stimulation. No expression changes were found in the control (unstimulated) cell group. **p<0.05 and ****p<0.0001.

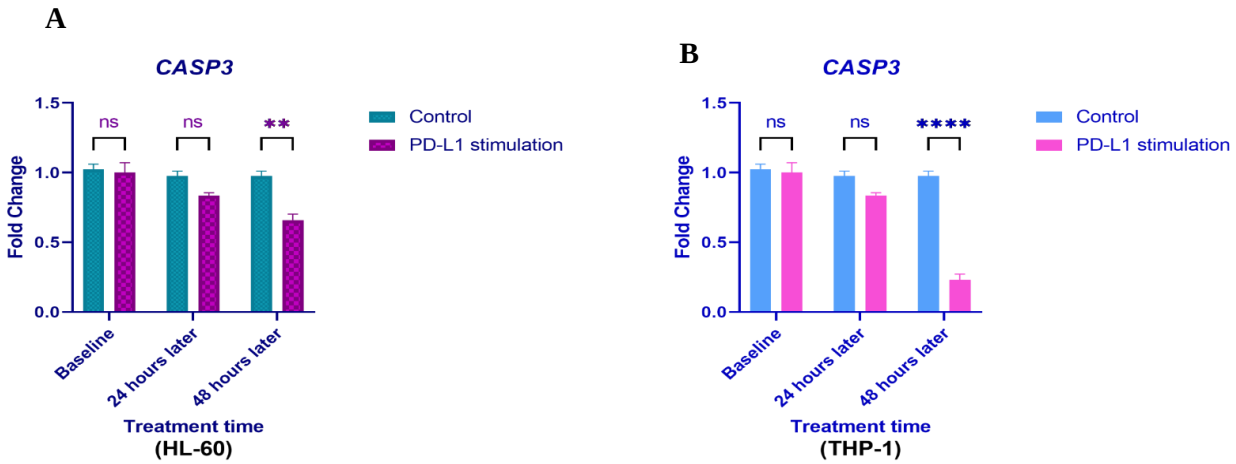

Figure 4. Expression changes of *CASP3* at baseline, 24 and 48 hours after PD-L1 stimulation in stimulated cells compared with control. A) In the HL-60 cell line, *CASP3* expression decreased after PD-L1 stimulation and most changes occurred 48 hours after treatment. No expression changes were found in the control (unstimulated) cell group. (B) In the THP-1 cell line *CASP3* expression decreased after PD-L1 stimulation and the expression changes were significant as early as 48 hours after stimulation. No expression changes were found in the control (unstimulated) cell group. **p<0.05; ****p<0.0001; ns, not significant.

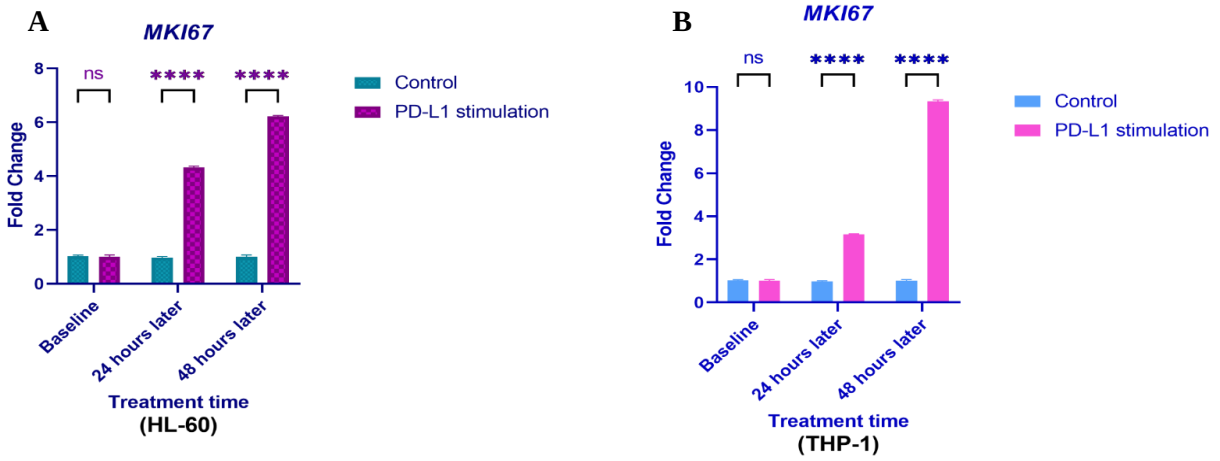

Figure 5. Expression of *MKI67* at baseline, 24 and 48 hours after PD-L1 stimulation. A) *MKI67* expression in the HL-60 cell line increased after PD-L1 stimulation B) *MKI67* expression in THP-1 cell line increased after PD-L1 stimulation. No expression changes were observed in the control (unstimulated) cells of either group. Most changes occurred 48 hours after treatment in both groups. ****p<0.0001.

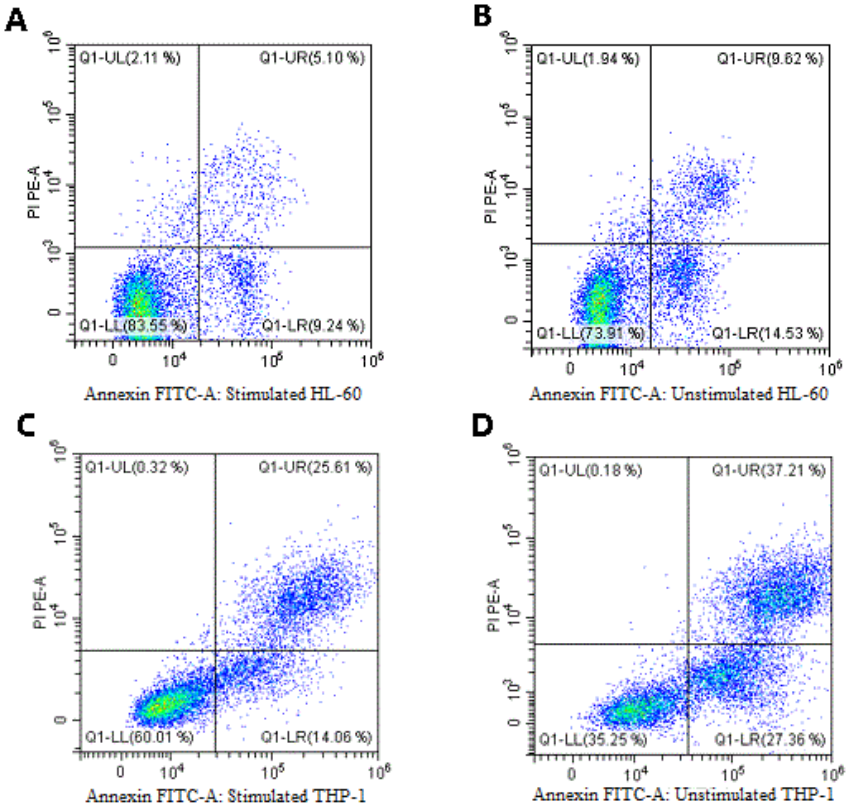

Figure 6. Annexin-V fluorescein isothiocyanate (FITC) labeling 72 hours after PD-L1 stimulation. A) PD-L1–stimulated HL-60 cells showed 14.34% annexin-V, while B) unstimulated controls displayed 24.15%. C) PD-L1–stimulated THP-1 cells exhibited 39.67% annexin-V, while D) unstimulated controls had 64.57%.

Table 1. Comparison of mean expression of proliferation, survival, and apoptosis between PD-L1–stimulated and control AML cell lines.

Genes	HL-60 Cells				THP-1 Cells			
	Control (Mean±SD)	Expression at baseline (Mean±SD)	Expression in 24 h (Mean±SD)	Expression in 48 h (Mean±SD)	Control (Mean±SD)	Expression in baseline (Mean±SD)	Expression in 24 h (Mean±SD)	Expression in 48 h (Mean±SD)
BCL2	1±0.05	1.03±0.27	5.095±0.28	11.635±1.18	1±0.05	0.975±0.14	1.33±0.11	7.06±1.03
BAX	1±0.05	1±0.13	0.67±0.15	0.375±0.07	1±0.05	1.025±0.22	0.795±0.09	0.96±0.31
CASP3	1±0.05	1.025±0.47	0.835±0.16	0.66±0.18	1±0.05	1.03±0.39	0.835±0.21	0.23±0.09
KI67	1±0.05	0.975±0.11	4.33±0.86	6.215±0.23	1±0.05	1±0.27	3.155±0.15	9.32±2.74

DISCUSSION

While PD-L1 and its biological actions in cancer cells have been investigated in several malignancies, research in the context of AML remains relatively limited. The present study focused primarily on the impact of PD-L1 on proliferation, survival, and apoptosis in AML cell lines.

Overexpression of PD-L1 has been identified in AML during conventional therapies or at relapse often coinciding with adverse clinical outcomes in these patients.[22] Dong et al, showed that PD-L1 expression in AML cells may promote Treg cell expansion, and the IL-35 or IL-10 produced by Tregs might stimulate AML cell proliferation.[12] Geduck et al, reported that PD-L1 appears to hold clinical significance as a prognostic factor, even in cases with low expression levels. In that study, a possible favorable prognostic effect of low PD-L1 expression on overall survival was observed.[23] While the importance of PD-L1 as a key extrinsic ligand for the immune checkpoint PD-1 is recognized, downstream signaling pathways of PD-L1, especially in hematologic malignancies are not well elucidated. Among the most important signaling pathways identified downstream of PD-L1 is the PI3K/Akt/mTOR pathway.[8,24]

In this study, we investigated the direct effect of PD-L1 stimulation through its recombinant PD-1 receptors on the proliferation, survival, and apoptosis of two human AML cell lines, HL-60 and THP-1. In addition, we investigated Bcl-2, a highly specific antiapoptotic protein, and the expression changes in *BCL2*, a widely recognized apoptosis suppressor gene.[25] Overexpression of Bcl-2 in cancer cells may block or delay apoptosis, leading to the selection and maintanance of long-lived cells and cell cycle arrest in the G0 phase.[14,26]

We also explored alterations in the expression of apoptosis regulators Bax and caspase-3 which play crucial roles in cell apoptosis.[27-30] In our study, PD-L1 stimulation promoted AML cell proliferation and survival. This effect was associated with an increase in the expression of Ki-67 and Bcl-2. Conversely, PD-L1 stimulation resulted in a reduction in cell apoptosis, as indicated by the decreased expression of Bax and caspase-3. Additionally, the flow cytometry assay revealed that stimulated cells exhibited lower annexin-V expression compared to control cells.

Our findings indicated that the changes in protein expression after PD-L1 stimulation were more prominent in HL-60 cell groups. This observation may be attributed to higher PD-L1 cell surface expression leading to stronger intracellular signaling. The most significant changes in expression were observed 48 hours after PD-L1 stimulation which may demonstrate the time needed for signaling, activation of transcription factors, and finally mRNA transcription.

Wang et al, found that PD-L1 controls the proliferation and apoptosis of leukemic cells in AML through activation of the PI3K-AKT-mTOR signaling pathway.[31] They used a series of bioinformatic methods with small interfering RNA (siRNA) and discovered that increased expression of PD-L1 was significantly correlated with worse patient survival, older age, and poor cytogenetics in patients with AML. Taken together, their results suggest that PD-L1 has a strong association with numerous genes that regulate cancer progression and prognosis.[32,33]

Our data were also consistent with findings in other malignancies in humans. Ghebeh et al. reported that in human breast cancer, elevated PD-L1 expression was associated with more proliferating cancer cells and that the overexpression of PD-L1 promoted tumor cell

growth.[17] Furthermore, Li et al, found that PD-L1 knockdown in gastric cancer cells could significantly curb cell proliferation, migration, and invasion, and promote apoptosis.[34] In contrast to these studies, we stimulated PD-L1 on the cell surface with its recombinant receptor to induce as similar a state as possible in the tumor microenvironment. Our results suggest that PD-L1 may regulate the biological functions of AML blast cells.

One of the main limitations of this study is associated with the culture of the THP-1 cell line, which is susceptible to microbial contamination. This is why considering sterile conditions during cell culture is imperative. This cell line also presented challenges in terms of growth and based on our findings, RPMI 1640 supplemented with 18% to 20% FBS may be the optimal culture conditions. Additionally, the use of PMA for cell stimulation introduced another level of complexity. A precise control of its concentration is essential to inhibit potential cytotoxic effects.

In summary, the present study demonstrated that stimulation with PD-L1 in human AML cell lines leads to an increase in cell proliferation and survival while concurrently reducing cell apoptosis in these cells.

STATEMENT OF ETHICS

The ethics approval code: IR.ARI.MUI.REC.1400.101.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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MS and BG designed the study, MS performed most of the experiments and analyzed the data. MV, HSH, PB, and MGH performed some of the experiments and statistical analysis. MS and NE wrote the original manuscript, and reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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